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Determination of Neuraminic Acid in Crude Brain Lipids

By C. LONG and D. A. STAPLES
Biochemistry Division, Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2

(*Received* 11 *May* 1959)

Strandin, a water-soluble lipid, was first isolated from brain by Folch, Arsove & Meath (1951). Recently, Bogoch (1958) has reported that purified ox-brain strandin (ganglioside) has a molecular weight of about 250 000. The molecule contains fatty acid, sphingosine, glucose, galactose, galactosamine and neuraminic acid (Klenk & Lauenstein, 1953; Bogoch, 1958).

In experiments designed to effect the chromatographic separation of cerebroside from crude brain lipids, the presence of strandin in the latter was considered to be a complicating factor, because of its related composition, and it became important to achieve the complete removal of the strandin before applying chromatography. For this purpose it was necessary to have a reliable method for the determination of strandin.

Neuraminic acid is the most characteristic constituent of strandin, for it has not been found in other lipids. Quantitative determination was therefore based on this component. In addition to the humin reaction described by Folch *et al.* (1951), which is not very sensitive, four other methods are available for its determination, namely reaction with (1) Bial's orcinol reagent, (2) Ehrlich's reagent, (3) diphenylamine and (4) tryptophan and perchloric acid (Werner & Odin, 1952). The sensitivites of reactions (1)–(4) are in the approximate ratio 5:1:2:1 respectively. Since the quantity of neuraminic acid in the lipid fraction extractable from brain amounts only to about 1·5–2·0 μmoles/g. of moist tissue, the very sensitive orcinol reaction has been used in the experiments to be described.

A preliminary report on this work has already been published (Long & Staples, 1959).

EXPERIMENTAL

Materials

Gifts of the following materials were used: synthetic *N*-acetylneuraminic acid (Cornforth, Firth & Gottschalk, 1958) from Miss Patricia Carroll; partially purified ox-brain strandin (Folch *et al.* 1951) from Dr G. H. Sloane-Stanley; ox-brain cerebroside, prepared by Dr R. Rodnight, from Dr G. H. Sloane-Stanley.

Methanol was purified by distillation from solid KOH. $CHCl_3$ was washed with water, dried over $CaCl_2$ and distilled; it was stabilized by the addition of 2% (v/v) of methanol. All other reagents were the best available commercial preparations.

Methods

Brain-lipid extracts. Ox brain, freshly obtained from the Metropolitan Cattle Market, was homogenized with $CHCl_3$–methanol (2:1, v/v) in the proportions of 1 g.:19 ml. The homogenate was centrifuged and the supernatant filtered. Assuming the brain to contain 80% of water, the final solvent composition would be: $CHCl_3$–methanol–water (approx. 64:32:4, by vol.). Extracts of rat tissues were prepared similarly.

Solvent-partition of lipids. This was done essentially as described by Folch, Lees & Sloane-Stanley (1957). To the lipid extract (150 ml.), in a 250 ml. centrifuge bottle fitted with a ground-glass stopper, 30 ml. of 0·1 M-KCl was added. The mixture was shaken and centrifuged. The upper aqueous methanolic phase (about 80 ml.) was transferred to a 250 ml. volumetric flask. The lower $CHCl_3$-rich phase was then extracted twice by shaking each time with 80 ml. of $CHCl_3$–methanol–0·1 M-KCl (3:48:47, by vol.), followed by centrifuging. The upper phases were combined and diluted to 250 ml. with methanol. The lower $CHCl_3$-rich phase was diluted to 200 ml. with methanol.

Orcinol reaction. This was carried out essentially according to Böhm, Dauber & Baumeister (1954). A measured volume of the test sample was evaporated to dryness in a 10 ml. centrifuge tube, and 1 ml. of water was added, followed by 1 ml. of Bial's orcinol reagent (0·02% of $FeCl_3$ + 0·2% of orcinol in 8 N-HCl). The mixture was heated on a boiling-water bath for 15 min., cooled and extracted with 4 ml. of amyl alcohol. After centrifuging, the upper violet layer was removed and a 3·5 ml. sample was transferred to a 1 cm. glass cell. Ethanol (0·05 ml.) was also added to the cell and the contents were mixed. The presence of the ethanol prevents the solution from becoming turbid owing to the absorption of moisture. Extinctions were measured at wavelengths between 400 and 700 mμ in a Unicam spectrophotometer, SP. 500. Standard *N*-acetylneuraminic acid and a reagent blank were simultaneously carried through the same procedure.

RESULTS

Direct orcinol reaction

Fig. 1 shows the absorption curve in amyl alcohol of the reaction product formed on heating 50 μg. (0·16 μmole) of synthetic *N*-acetylneuraminic acid with Bial's orcinol reagent. The two characteristic features of this curve are the absorption maximum at 570 mμ (ϵ, $6{\cdot}0 \times 10^3$) and the flat portion between

440 and 480 mμ (ϵ, $3{\cdot}8 \times 10^3$). The latter probably represents the summation of two curves, namely the absorption due to the orcinol-reaction product and that due to a part of the neuraminic acid which undergoes decomposition on heating with HCl alone (Folch *et al.* 1951) to give a brown pigment with absorption maximum at about 420 mμ. The partially purified specimen of ox-brain strandin (165 μg.) gave a curve almost identical in shape with that of *N*-acetylneuraminic acid (Fig. 1).

When the orcinol reaction was applied to the total lipids extractable from 75 mg. of ox brain, the absorption curve (Fig. 1) showed two peaks at 425 and 560 mμ and a pronounced shoulder at about 650 mμ. From this it is clear that the total lipids contain substances other than neuraminic acid which react with the orcinol reagent and that no quantitative conclusions can be drawn about the neuraminic acid content.

Folch *et al.* (1957) reported that when a solution of total brain lipids in $CHCl_3$–methanol–water (64:32:4, by vol.) was shaken with water or a dilute aqueous salt solution (0·2 vol.), the resulting upper aqueous methanolic phase contained strandin. The ox-brain lipid solution was therefore treated with 0·1 M-KCl and partitioned into upper (aqueous methanolic, *A*) and lower ($CHCl_3$-rich, *B*) phases, as described in the Methods section. When amounts of each phase, derived from 75 mg. of ox brain, were separately subjected to the orcinol reaction, the results shown in Fig. 2 were obtained. The curve for the upper-phase material (*A*) somewhat resembled those obtained with synthetic *N*-acetylneuraminic acid and ox-brain strandin (Fig. 1), but the existence of the minor peak at 440–450 mμ, instead of the flat portion in this region, and the inflexion at 650 mμ indicated the presence of small amounts of orcinol-reactive material other than neuraminic acid. From this it can be seen that the determination of the neuraminic acid content of the upper phase (*A*) from the extinction at 570 mμ would probably lead to an erroneously high value.

The lower-phase material (*B*) was also orcinol-reactive (Fig. 2), showing a maximum at 415 mμ, but with some absorption in the region of 570 mμ. It seemed probable that this behaviour was largely due to the presence, in the lower phase, of cerebroside, which on analysis amounted to 2·30 μmoles, since similar types of curves (Fig. 2) were obtained with 1·5 μmoles of cerebroside or galactose.

Effect of acid treatment before applying the orcinol reaction

As previously mentioned, Folch *et al.* (1951) developed a method for the estimation of strandin whereby the lipid sample was heated with conc. HCl and the resulting brown decomposition product

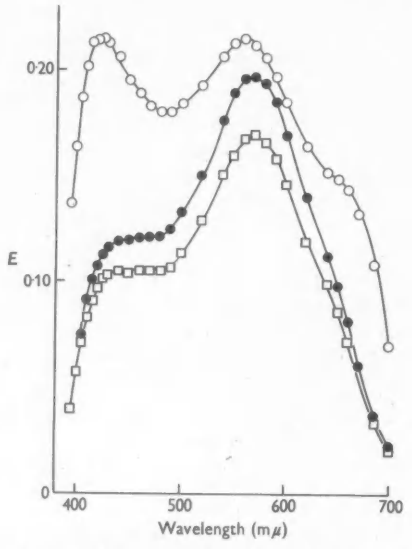

Fig. 1. Absorption spectra of orcinol-reaction products in amyl alcohol. ●, 0·16 μmole of synthetic *N*-acetylneuraminic acid; □, 165 μg. of partially purified ox-brain strandin; ○, mixed ox-brain lipids, derived from 75 mg. of moist tissue. Total vol., 4·8 ml.; 1 cm. light path.

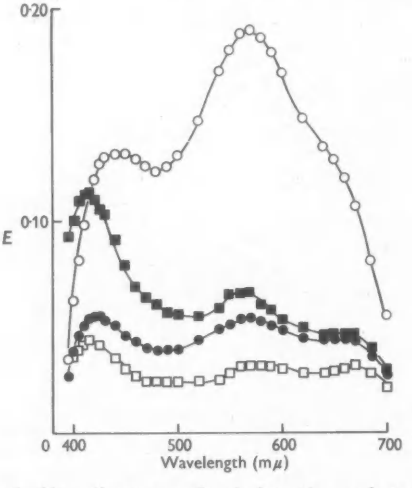

Fig. 2. Absorption curves of orcinol-reaction products in amyl alcohol. ○, Upper aqueous methanolic phase derived from 75 mg. of moist ox brain; ■, lower $CHCl_3$-rich phase derived from 75 mg. of moist ox brain; ●, cerebroside (1·5 μmoles); □, galactose (1·5 μmoles).

was determined spectrophotometrically after dilution with acetone. When synthetic *N*-acetylneuraminic acid was first heated with either acid or alkali and the orcinol reaction subsequently applied, extensive decomposition was found to have taken place (Fig. 3). The sample of ox-brain strandin behaved similarly under acidic conditions, but was completely stable to treatment with hot alkali. These findings suggest that only free neuraminic acid is susceptible to alkaline decomposition, and that the stability of strandin is due to the alkali-resistance of the glycosidic bond which links neuraminic acid to the rest of the molecule. It will also be seen from Fig. 3 that the characteristic change, produced by prior treatment with HCl on the orcinol reaction of neuraminic acid, is the fall in extinction at 570 mμ.

In order to determine whether the lower-phase material (*B*) from the partition experiment (Folch *et al.* 1957) contained any neuraminic acid, use was made of this acid-lability of neuraminic acid derivatives. A sample of lower-phase material (*B*), corresponding to 75 mg. of moist ox brain, was evaporated to dryness and the lipid residue was heated with 1 ml. of 4N-HCl on a boiling-water bath for 30 min. After cooling, the contents of the tube were extracted with a mixture of methanol (1·6 ml.) and $CHCl_3$ (3·2 ml.). The upper aqueous methanolic phase which separated on standing was discarded. The lower phase was extracted twice with 2 ml. portions of $CHCl_3$–methanol–water (3:48:47, by vol.) and the upper phases were again discarded. The lower $CHCl_3$-rich phase (*C*) was then evaporated to dryness and treated with the orcinol reagent in the usual way. An equivalent sample of the original lower phase (*B*), which had not been heated with acid, was also subjected to the orcinol reaction. The results of this experiment are shown in Fig. 4. Although there are slight differences in the extinctions below 470 mμ and above 590 mμ, the curves are superimposable between these

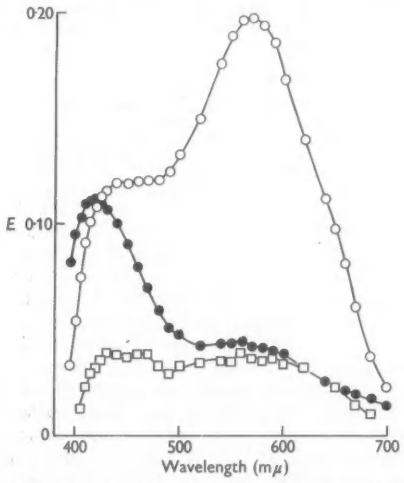

Fig. 3. Effect of heating *N*-acetylneuraminic acid with acid or alkali on the absorption spectra of the orcinol-reaction products. All samples originally contained 0·16 μmole of *N*-acetylneuraminic acid. ○, Untreated; ●, preliminary treatment with 4N-HCl at 100° for 30 min.; □, preliminary treatment with N-NaOH at 100° for 30 min.

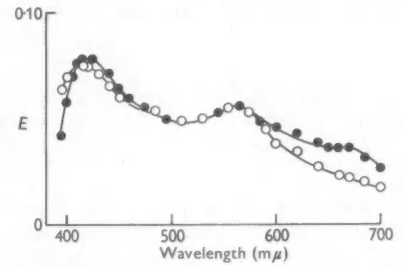

Fig. 4. Absorption spectra of orcinol-reaction products of $CHCl_3$-rich phase derived from 75 mg. of moist ox brain before and after heating with 4N-HCl at 100° for 30 min. ●, Untreated; ○, after hot-acid treatment.

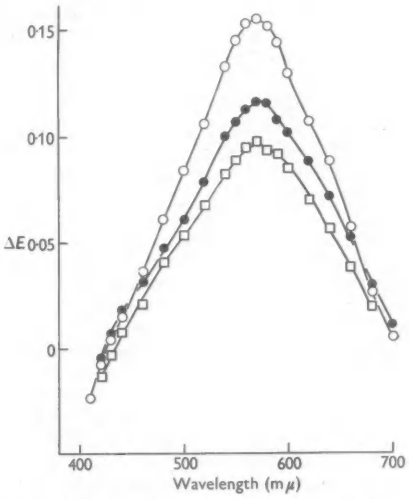

Fig. 5. 'Difference' absorption spectra of orcinol-reaction products (untreated minus acid-treated). ○, *N*-Acetylneuraminic acid (0·16 μmole); □, partially purified strandin (135 μg.); ●, upper aqueous methanolic phase derived from 75 mg. of moist ox brain.

Table 1. *Neuraminic acid content of lipid extracts of rat tissues*

Tissues were homogenized with $CHCl_3$–methanol (2:1, v/v) and determinations made as described in text.

Tissue	Difference extinction (ΔE_{570}/75 mg. of fresh tissue)	*N*-Acetylneuraminic acid (μmoles/g. of fresh tissue)
Brain (grey matter)	0·118, 0·137	1·54, 1·83
Brain (white matter)	0·072, 0·068	0·94, 0·90
Brain (cerebellum)	0·066, 0·078	0·86, 1·04
Lung	0·003	0·04
Skeletal muscle	0·002	0·02
Kidney	0·002	0·02
Uterus	0·002	0·02
Spleen	0·001	0·01
Adipose tissue	0·001	0·01
Heart	0	0
Intestine (small)	0	0
Liver	0	0

wavelengths. The absence of any fall in extinction at 570 mμ, as a result of the hot-acid treatment, has been taken to show conclusively that the lower-phase material (*B*) was free from neuraminic acid.

Since it follows that the whole of the neuraminic acid content of the original lipid extract must be present in the upper phase (*A*), it only remained to develop a suitable method for its determination. This has also been achieved by making use of the selective destruction of neuraminic acid during a preliminary acid hydrolysis. In Fig. 5 are shown the 'difference' curves obtained with synthetic *N*-acetylneuraminic acid, partially purified ox-brain strandin and the upper-phase material (*A*) from 75 mg. of ox brain. The difference extinctions (ΔE) were obtained by deducting the extinction of an acid-treated sample from that of an equal amount of an untreated sample, after both had been subjected separately to the orcinol reaction. It will be seen from Fig. 5 that there are no important differences between the shapes of these three curves, all of which show maxima at 570 mμ. When different concentrations of strandin or *N*-acetylneuraminic acid were used, the ΔE values at 570 mμ were always directly proportional to concentration, at least up to 0·16 μmole of neuraminic acid, which was the highest concentration tested. From these curves, the partially purified ox-brain strandin was calculated to be 68% pure, assuming that strandin contains 30·9% of neuraminic acid (Bogoch, 1958). Similarly, ox-brain lipid was calculated to contain 1·64 μmoles of *N*-acetylneuraminic acid/g. of moist tissue.

It has been found that neuraminic acid determinations, based on this same principle, can also be made equally well with a simple photoelectric colorimeter of the EEL (Evans Electroselenium Ltd.) type with a no. 626 filter in place of a spectrophotometer.

Neuraminic acid content of lipid extracts of rat tissues

Folch *et al.* (1951) reported that tissues other than brain contained little or no strandin, but the method of determination used by these workers, the humin reaction, was relatively insensitive. With the more sensitive method now available, we have redetermined the neuraminic acid content of lipid extracts from several rat tissues.

A sample (0·2 g.) of fresh tissue was homogenized in a Potter–Elvehjem-type homogenizer with 3·8 ml. of $CHCl_3$–methanol (2:1, v/v) and the suspension was centrifuged. The supernatant was transferred to a 50 ml. distillation flask. The residue was washed twice with 2 ml. of solvent and the washings were combined with the original supernatant. After evaporating the extract to dryness, the lipid residue was dissolved in about 2 ml. of $CHCl_3$–methanol (2:1, v/v). Any insoluble material was removed by filtration, and the filtrate and washings were diluted to 3 ml. This lipid extract was then partitioned by the method of Folch *et al.* (1957), as described in the Methods section. Two samples of upper phase, each corresponding with 75 mg. of moist tissue, were taken for determination of the difference extinction at 570 mμ, after treatment with the orcinol reagent. The results obtained (Table 1) confirm that neuraminic acid is present only in lipid extracts of brain. Grey matter from the cortex contained about twice the amount of *N*-acetylneuraminic acid, on a moist-weight basis, as did white matter or cerebellum.

DISCUSSION

The primary aim of the work described was to effect the complete removal of strandin from crude brain lipids, with a view to carrying out sub-

sequent chromatographic separations of the individual constituents. The partitioning of brain lipids between aqueous methanolic and chloroform-rich phases, according to the method of Folch *et al.* (1957), resulted in the complete transfer of the strandin into the upper phase. In unpublished work, we have shown that negligible amounts of other lipids are lost in this way. However, the water-soluble non-lipid impurities do pass into the upper phase (cf. also Folch *et al.* 1957). The carbohydrate content is about 30% greater than would be expected from the composition of strandin (Bogoch, 1958), and this excess probably represents water-soluble sugars and their derivatives.

The presence of this extra carbohydrate in the upper phase almost certainly accounts for the extra peak at 440 mμ (Fig. 2), after the orcinol reaction. Although small in absolute amount, this carbohydrate would also contribute significantly to the absorption at 570 mμ, so giving an erroneously high value for the neuraminic acid content based on direct readings of E_{570}. The use of 'difference' extinctions, depending on the selective destruction of neuraminic acid by hot-acid treatment, satisfactorily removes this interference.

SUMMARY

1. Synthetic *N*-acetylneuraminic acid and partially purified ox-brain strandin have been subjected to Bial's orcinol reaction and the characteristics of the absorption spectra have been studied.

2. Crude ox-brain lipids under similar conditions showed anomalous spectra, which were largely traced to the presence of cerebroside.

3. Partitioning of ox-brain lipids between aqueous methanolic and chloroform-rich phases caused the strandin to pass completely into the upper aqueous methanolic phase. The neuraminic acid content of this phase was determined by measuring the 'difference' extinction at 570 mμ of the orcinol-reaction products (untreated minus acid-treated).

4. Neuraminic acid has been determined in the lipids of several rat tissues. It was found only in the brain.

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Metabolism of Polycyclic Compounds

14. THE CONVERSION OF NAPHTHALENE INTO COMPOUNDS RELATED TO *TRANS*-1:2-DIHYDRO-1:2-DIHYDROXYNAPHTHALENE BY RABBITS*

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(*Received* 29 *April* 1959)

The conversion of aromatic hydrocarbons into dihydrodihydroxy compounds by 'perhydroxylation' was first recognized when Boyland & Levi (1935, 1936) isolated (+)- and (−)-1:2-dihydro-1:2-dihydroxyanthracene, and the corresponding glucosiduronic acids, from the urine of rabbits and rats respectively which had been dosed with anthracene. Boyland & Wolf (1950) showed that phenanthrene is converted by rats into *trans*-9:10-dihydro-9:10-dihydroxyphenanthrene and by rabbits into this diol and into (−)-*trans*-1:2-dihydro-1:2-dihydroxyphenanthrene. Young (1947) found that naphthalene is converted by rats into (−)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene and Booth & Boyland (1949) obtained both the (±) and the (−)-forms of this diol from rabbits treated with naphthalene. It has been shown that naphthalene is converted into 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid in rabbits (Corner, Billett & Young, 1954) and in rats (Corner & Young, 1954). The product from rabbits yielded (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene on hydrolysis

* Part 13: Booth & Boyland (1958).

with β-glucuronidase, whereas that from rats yielded a substance which was a mixture of the (±)- and the (−)-forms of the diol.

The present work has shown that naphthalene is converted by rabbits into the 1-glucosiduronic acids of both (+)- and (−)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene and into the 2-glucosiduronic acid of at least one of these forms. Evidence for the formation of 1-hydroxy-2-naphthyl sulphate by rabbits treated with either naphthalene or with (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene is also presented.

EXPERIMENTAL

Melting points are uncorrected.

Paper chromatography. This was carried out on Whatman no. 1 chromatography paper. Descending chromatograms were developed with solvent 1 or 2 (Table 1) for 15 hr. and ascending chromatograms with solvent 3 (Table 1) for 5 hr. The chromatograms were dried at room temperature, examined under u.v. light after exposure to NH_3 and then sprayed with a solution of freshly diazotized *p*-nitroaniline (0·02% in 0·1 N-HCl), followed by aq. 10% Na_2CO_3 soln. either immediately, or after being sprayed with aq. 2 N-HCl soln. and heated in an oven to 70°. On chromatograms heated with acid, compounds which had given rise to 1-naphthol and 1:2-dihydroxynaphthalene appeared as purple and blue spots respectively without further treatment.

(±)-1:2-*Dihydro*-1:2-*dihydroxynaphthalene.* This compound was prepared by the method of Booth, Boyland & Turner (1950) and formed silky needles, m.p. 101°.

(−)-1:2-*Dihydro*-1:2-*dihydroxynaphthalene.* This compound was isolated from the urine of rats dosed with naphthalene as described below for the (+)-isomer and formed plates from benzene, m.p. 128°, $[\alpha]_D^{22}$ −157° (*c*, 0·5 in ethanol). It formed a diacetate, m.p. 77°, $[\alpha]_D^{22}$ −435° (*c*, 0·5 in ethanol).

Potassium 1-hydroxy-2-naphthyl sulphate. 1-Naphthol (10 g.) was oxidized with $K_2S_2O_8$ (20 g.) as described for 2-naphthol (Boyland & Sims, 1957). The oxidation products eluted from charcoal with methanol were shown on paper chromatograms to contain two substances with R_F values of 0·40 and 0·45 respectively in solvent 1 (see Table 1), and which gave purple and blue colours respectively with diazotized *p*-nitroaniline and aq. Na_2CO_3 soln. The products were chromatographed on a cellulose-powder column [prepared from cellulose powder (250 g.) as described previously (Boyland & Sims, 1958)] with butanol–*cyclo*hexane–aq. 2 N-NH_3 soln. (9:2:1, by vol.) as the developing solvent, and the eluate, collected in fractions of 250 ml., was examined on paper chromatograms. The fractions in which the substance of R_F 0·45 was predominant were combined and evaporated and the residue was rechromatographed on a smaller cellulose-powder column. The fractions rich in this substance were combined, a small excess of aq. 2 N-KOH was added and the solution evaporated. The residue was recrystallized several times from aq. ethanol to yield *potassium 1-hydroxy-2-naphthyl sulphate* (56 mg.) in pale-pink plates (Found: S, 11·3. $C_{10}H_7O_5SK$ requires S, 11·5%). A little of the ester was heated to 100° with 5 N-HCl (0·1 ml.) for 15 min. and the product which separated on cooling was acetylated with acetic anhydride in pyridine to yield the diacetate of 1:2-dihydroxynaphthalene, m.p. and mixed m.p. 108–109°.

The second product from the oxidation, which was not isolated, is presumed to be 4-hydroxy-1-naphthyl sulphate. Desai & Sethna (1951) isolated 1:4-dihydroxynaphthalene from the persulphate oxidation of 1-naphthol after the reaction mixture had been heated with mineral acid.

Dosing of animals. The methods used in the dosing of animals, in the collection of urine and in the treatment of the urine with charcoal have already been described (Boyland & Sims, 1958).

Table 1. *Paper chromatography of* trans-1:2-*dihydroxynaphthalene and related compounds*

Solvent systems: 1, butanol saturated with aq. 2 N-NH_3 soln. (downward development); 2, butanol–propan-1-ol–aq. 2 N-NH_3 soln. (2:1:1, by vol.; downward development); 3, aq. 0·1 N-NH_3 soln. (upward development). The colours in parentheses refer to those obtained after the chromatograms had been treated with 2 N-HCl (see text).

Metabolite	R_F in solvent 1	2	3	Fluorescence	Colour with diazotized *p*-nitroaniline
1-Naphthol	0·96	0·97	0·62	Blue	Blue
2-Naphthol	0·96	0·97	0·55	Violet	Orange
trans-1:2-Dihydro-1:2-dihydroxynaphthalene	0·86	0·89	—	Dark absorption	— (blue)
1-Naphthyl sulphate	0·51	0·62	—	Dark blue	Violet (blue)
2-Hydroxy-1-naphthyl sulphate	0·49	0·58	—	Light blue	Brown* (purple)
1-Hydroxy-2-naphthyl sulphate	0·46	0·55	—	Light blue	Blue (purple)
2-Naphthyl sulphate	0·46	0·53	—	Dark blue	— (orange)
1-Naphthylglucosiduronic acid	0·11	0·28	—	Dark blue	— (blue)
1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid†	0·08	0·18	—	Dark absorption	Pale orange‡ (blue)
1:2-Dihydro-1-hydroxy-2-naphthylglucosiduronic acid	0·08	0·18	—	Dark absorption	Pale blue

* After 24 hr.

† The optically active forms could not be distinguished on paper chromatograms.

‡ These colours are presumably those of the naphthols produced by the decomposition of the glucosiduronic acids. 1:2-Dihydro-1-hydroxy-2-naphthylglucosiduronic acid is more readily detected on two-dimensional chromatograms (see text).

Treatment of urine extracts. This procedure, with cellulose-powder columns, was described by Boyland & Sims (1958) and an indication of when the various metabolites of naphthalene would be eluted from such columns was also given. Not all the naphthalene metabolites were eluted from charcoal by methanol–NH_3. Paper chromatography showed that some 1-naphthylmercapturic acid precursor and some 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acids were eluted when the charcoal was subsequently washed with aq. phenol (5%, w/v). The separations described below are confined to the material eluted from the charcoal with methanol–NH_3.

Separation of the various metabolites on cellulose-powder Columns. The separations were carried out on the products obtained as indicated above when six rabbits were given a total of 30 g. of naphthalene during 5 days. The first two fractions from the column were evaporated under reduced pressure to a brown gum, which was extracted several times with boiling light petroleum (b.p. 80–100°). The solvent was removed under reduced pressure and the extracted solid recrystallized from water to yield 2-naphthol in plates, m.p. and mixed m.p. 120–121°. Yields of 110, 150 and 120 mg. of 2-naphthol were obtained from three separate experiments. 1-Naphthol could not be isolated, although it was detected by paper chromatography.

Fractions containing *trans*-1:2-dihydro-1:2-dihydroxynaphthalene were combined and evaporated, and the residual gum was extracted several times with boiling *cyclo*hexane. The product which separated on cooling was usually a mixture of the optically active forms of the compound with the (+)-form predominating: after one recrystallization from benzene, a typical specimen had m.p. 110–112°, $[\alpha]_D^{22}$ +68° (*c*, 1 in ethanol). After this had been recrystallized three times from benzene, the (+)-form was obtained, m.p. 127°, $[\alpha]_D^{22}$ +158° (*c*, 1 in ethanol), which formed a diacetate in plates (from aqueous methanol), m.p. 78°, $[\alpha]_D^{20}$ +430° (*c*, 0·5 in ethanol). Evaporation of the benzene mother liquors gave a solid which was recrystallized from benzene to yield (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene in silky needles, m.p. 101°. Yields of the dihydrodihydroxy compound were variable: 520 and 110 mg. were obtained from two separate experiments. On a third occasion none was isolated but paper chromatography showed the presence of traces in the untreated urine. On one occasion only the (±)-form was obtained.

Sulphuric acid esters of 1:2-*dihydroxynaphthalene.* Fractions containing these esters also contained 1-naphthyl sulphate: they were combined, evaporated and treated with aq. NaOH as described by Boyland & Sims (1957) to yield the sodium salt of 2-hydroxy-1-naphthyl sulphate. The mother liquors, when examined on paper chromatograms, were seen to contain this sulphate, together with 1-naphthyl sulphate and a substance which had the properties of 1-hydroxy-2-naphthyl sulphate. Attempts to isolate this last substance were unsuccessful.

Glucosiduronic acid derivatives of trans-1:2-*dihydro*-1:2-*dihydroxynaphthalene.* After fractions containing the 1-naphthylmercapturic acid precursor (Boyland & Sims, 1958) had been collected, elution of the column was continued and a number of fractions were obtained which were shown on paper chromatograms to contain compounds which gave naphthol reactions after the paper was heated with acid. During the evaporation of some of the earlier of these fractions a crystalline compound was obtained which gave rise on paper chromatograms to two spots, one of which gave 1-naphthol reactions after acid hydrolysis. The second spot was identical with that of creatinine. Consideration of the evidence presented below shows the compound to be the *creatinine salt* (or complex) of (−)-*trans*-1:2-dihydro-2-hydroxy-1-naphthyl-D-glucosiduronic acid: from aq. ethanol it formed elongated plates, m.p. 238–239° (decomp.), $[\alpha]_D^{20}$ −233° (*c*, 0·5 in water) (Found: C, 53·5; H, 5·9, N, 9·3. $C_{20}H_{25}O_9N_3$ requires C, 53·2; H, 5·6; N, 9·3%). On one occasion it was found that the naturally occurring creatinine was eluted from the column before the glucosiduronic acids. The fractions containing these acids were therefore combined and evaporated to a small volume and creatinine (2 g.) was added. The mixture was heated to 100° for a few minutes and cooled, when the creatinine salt described above separated.

The later fractions from the cellulose-powder column were evaporated to yield gums which gave strongly positive naphtharesorcinol tests for glucosiduronic acids. These fractions and the evaporated mother liquors from the earlier fractions were combined and re-chromatographed on a smaller cellulose column with butanol–*cyclo*hexane–aq. 2 N-NH_3 soln. (22:2:3). Fractions shown by paper chromatography to contain the glucosiduronic acids of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene were combined and evaporated to dryness under reduced pressure, and the gum thus obtained was dissolved in water (100 ml.). The aqueous solution was passed through a layer of the ion-exchange resin Zeo-Karb 225 (in the acid form) and evaporated to dryness under reduced pressure. The residue was recrystallized from ethanol–light petroleum (b.p. 80–100°) to yield a colourless powder, m.p. 146–158° (decomp.) which was raised to 152–153° by several recrystallizations from the above-mentioned solvents. It appears from evidence presented below that the compound is mainly (+)-*trans*-1:2-dihydro-2-hydroxy-1-naphthyl-D-glucosiduronic acid, $[\alpha]_D^{20}$ +50° (*c*, 0·5 in ethanol) (Found: C, 56·4; H, 5·8. Calc. for $C_{16}H_{18}O_8$: C, 56·8; H, 5·4%).

Also detected in the glucosiduronic acid fractions by means of paper chromatography were 1-naphthylglucosiduronic acid and two substances believed to be the glucosiduronic acid derivatives of 1:2-dihydroxynaphthalene. The structures of the two last-named compounds, which were present in traces in rabbit urine but in much greater amount in the urine of animals of other species, will be discussed in a later paper.

Examination of the creatinine salt of the glucosiduronic acid. A solution of the salt (600 mg.) in water (50 ml.) was passed through a layer of Zeo-Karb 225 (in the acid form). The solution was evaporated to dryness under reduced pressure to yield (−)-trans-1:2-*dihydro*-2-*hydroxy*-1-*naphthyl*-D-*glucosiduronic acid* (425 mg.), which was recrystallized from ethanol–light petroleum (b.p. 80–100°) in needles, m.p. 172–173° (decomp.), $[\alpha]_D^{22}$ −171 ± 5° (*c*, 0·5 in ethanol) (Found: C, 56·5; H, 5·25. $C_{16}H_{18}O_8$ requires C, 56·8; H, 5·4%). The ion-exchange resin was then eluted with aq. 2 N-NH_3 soln. and the eluate was evaporated to dryness and the residue recrystallized from aq. ethanol to yield creatinine (102 mg.), m.p. 260° (decomp.) (Found: N, 37·3. Calc. for $C_4H_7ON_3$: N, 37·15%). The picrate formed yellow needles from water, m.p. and mixed m.p. 217–218° (decomp.) (Found: N, 24·6. Calc. for $C_{10}H_{10}O_8N_6$: N, 24·6%).

The glucosiduronic acid (100 mg.) was heated to 60° with 2 N-HCl (2 ml.) for 15 min. and the solution was cooled and

extracted with ether. The ethereal layer was shown by paper chromatography to contain 2-naphthol. The aqueous layer was treated with charcoal (1 g.) and the charcoal was filtered off, washed with water and eluted with methanol (50 ml.) containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88). Removal of the methanol under reduced pressure afforded a gum which was shown by paper chromatography to contain 1-naphthylglucosiduronic acid. The gum was esterified with diazomethane in ether and acetylated with acetic anhydride in pyridine to yield methyl (1-naphthyltri-*O*-acetyl-D-glucosid)uronate (110 mg.), separating from aq. ethanol in needles, m.p. and mixed m.p. 158–159°. When the glucosiduronic acid was heated to 100° with 10 N-HCl for 30 min. and the products were examined on paper chromatograms only 1- and 2-naphthol were detected.

The glucosiduronic acid (100 mg.) was incubated with β-glucuronidase (Ketodase, supplied by Warner–Chilcott Laboratories; 10 ml.) at 37° for 24 hr. The solution was extracted twice with ether, the ether was evaporated and the product was recrystallized from benzene to yield (−)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (32 mg.) in plates, m.p. and mixed m.p. 127°, $[\alpha]_D^{22} - 157°$ (*c*, 0·5 in ethanol). The diacetate formed plates from light petroleum (b.p. 60–80°), m.p. and mixed m.p. 77°.

The glucosiduronic acid was esterified and acetylated as before to yield (−)-*methyl* (trans-2-*acetoxy*-1:2-*dihydro*-1-*naphthyltri-O-acetyl*-D-*glucosid*)*uronate*, separating from ethanol in needles, m.p. 194°, $[\alpha]_D^{22} - 293 \pm 5°$ (*c*, 0·5 in $CHCl_3$) (Found: C, 58·0; H, 5·6. $C_{25}H_{28}O_{12}$ requires C, 57·7, H, 5·4%).

Separation of the tetra-acetyl methyl ester derivatives of the glucosiduronic acids of 1:2-*dihydro*-1:2-*dihydroxynaphthalene.* In one experiment the fractions containing the glucosiduronic acids were combined and evaporated and the gum, in methanol, was treated with an excess of diazomethane. The solution was evaporated to dryness and the residue dissolved in pyridine and an excess of acetic anhydride was added. After some hours the reaction mixture was poured into a large volume of water and the product which separated was dissolved in a minimum amount of boiling ethanol and the solution allowed to crystallize. The crystals (850 mg.) were recrystallized from ethanol to yield (+)-*methyl* (trans-2-*acetoxy*-1:2-*dihydro*-1-*naphthyltri*-*O*-*acetyl*-D-*glucosid*)-*uronate* in long needles, m.p. 209–210°, $[\alpha]_D^{22} + 100 \pm 5°$ (*c*, 0·5 in $CHCl_3$) (Found: C, 57·4; H, 5·7. $C_{25}H_{28}O_{12}$ requires C, 57·7; H, 5·4%).

The mother liquors were evaporated to a small volume and the solution was allowed to crystallize. The product which separated was recrystallized three times from ethanol to yield (−)-methyl (*trans*-2-acetoxy-1:2-dihydro-1-naphthyltri-*O*-acetyl-D-glucosid)uronate (280 mg.) in needles, m.p. 194°, undepressed in a mixture with the product obtained earlier (Found: C, 57·9; H, 5·5. Calc. for $C_{25}H_{28}O_{12}$: C, 57·7; H, 5·4%). The combined mother liquors were evaporated to dryness and the residue was recrystallized twice from aq. ethanol and then from ethanol to yield a product which is believed to be (−)-*methyl* (1-*acetoxy*-1:2-*dihydro*-2-*naphthyltri*-*O*-*acetyl*-D-*glucosid*)-*uronate* (115 mg.) in needles, m.p. 168°, $[\alpha]_D^{22} - 228 \pm 5°$ (*c*, 0·5 in $CHCl_3$) (Found: C, 57·5; 57·9; H, 5·3, 5·7. $C_{25}H_{28}O_{12}$ requires C, 57·7; H, 5·4%).

Hydrolysis of (+)-*methyl*(2-*acetoxy*-1:2-*dihydro*-1-*naphthyltri*-*O*-*acetyl*-D-*glucosid*)*uronate.* The derivative (650 mg.), in a solution of barium methoxide in methanol (52 ml.; 0·125 N) (Bollenback, Long, Benjamin & Lindquist, 1955), was kept overnight at room temperature and the precipitate was collected, dissolved in water (250 ml.) and the solution passed through a layer of Zeo-Karb 225 (in the acid form). The solution was evaporated under reduced pressure and the product was recrystallized from ethanol–light petroleum (b.p. 80–100°) to give (+)-trans-1:2-*dihydro*-2-*hydroxy*-1-*naphthyl*-D-*glucosiduronic acid* (380 mg.) in needles, m.p. 151–152°, $[\alpha]_D^{22} + 106 \pm 2°$ (*c*, 0·5 in ethanol) (Found: C, 56·7; H, 5·8. $C_{16}H_{18}O_8$ requires C, 56·8; H, 5·4%). Esterification and acetylation as before yielded the original derivative, m.p. and mixed m.p. 209–210°.

The glucosiduronic acid (100 mg.) was heated with 2 N-HCl (2 ml.) at 60° for 15 min. and the products were treated with charcoal as described above. 2-Naphthol was detected in the methanol eluate by paper chromatography and when the gum obtained by evaporation of the methanol was treated with diazomethane followed by acetic anhydride in pyridine, methyl (1-naphthyltri-*O*-acetyl-D-glucosid)-uronate (98 mg.), m.p. and mixed m.p. 158–159°, was obtained.

Hydrolysis of the glucosiduronic acid with 10 N-HCl at 100° afforded 1- and 2-naphthol (detected on paper chromatograms). When the glucosiduronic acid (100 mg.) was hydrolysed with β-glucuronidase as previously described, (+)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (28 mg.) was obtained, separating from benzene in plates, m.p. and mixed m.p. 127°, $[\alpha]_D^{22} + 156°$ (*c*, 0·5 in ethanol). The diacetate formed plates from light petroleum (b.p. 60–80°), m.p. and mixed m.p. 77°.

Hydrolysis of the derivative of m.p. 168°. The derivative (70 mg.) was hydrolysed with barium methoxide in methanol (5·6 ml.; 0·125 N) as before to yield finally a colourless powder which appeared to consist mainly of *trans*-1:2-dihydro-1-hydroxy-2-naphthylglucosiduronic acid. A little of the acid was heated to 60° with 2 N-HCl for 15 min. and the products were examined on paper chromatograms. 1-Naphthol was detected but no 2-naphthylglucosiduronic acid appeared to have been formed. Hydrolysis of the acid with 10 N-HCl at 100° for 15 min. also afforded only 1-naphthol, and examination on paper chromatograms of the products of the hydrolysis with β-glucuronidase showed the presence of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene. When the compound was examined on two-dimensional chromatograms developed with either solvent 1 or 2, which was sprayed with 2 N-HCl between the first and second runs, some decomposition occurred to yield 1-naphthol: the formation of 1-naphthol in this way was used to detect the presence of the glucosiduronic acid in urine.

Metabolism of compounds other than naphthalene. Two rats were each injected intraperitoneally with 5 ml. of an aqueous solution of sodium 2-hydroxy-1-naphthyl sulphate (10%, w/v) and the urine was collected for 24 hr. after the injection. Examination of the urine on paper chromatograms showed the presence of unchanged 2-hydroxy-1-naphthyl sulphate, together with a little of the isomeric 1-hydroxy-2-naphthyl sulphate.

A rabbit, a rat and a guinea pig each was injected intraperitoneally with 5, 0·5 and 2 ml. respectively of a solution of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene in arachis oil (10%, w/v) and the urines were collected for 24 hr. after the injections. When the urines were examined on one- and two-dimensional paper chromatograms it was seen that in

each case 1-hydroxy-2- and 2-hydroxy-1-naphthyl sulphate and 1-naphthyl sulphate were present, together with 2-naphthol, 1-naphthylglucosiduronic acid, 1:2-dihydro-2-hydroxy-1-naphthyl- and 1:2-dihydro-1-hydroxy-2-naphthyl-glucosiduronic acid and unchanged *trans*-1:2-dihydro-1:2-dihydroxynaphthalene.

A rat was given a solution of (−)-*trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid (500 mg.) in water (2 ml.) by mouth. The urine was collected for the 24 hr. after treatment and examined on paper chromatograms. The urine contained 2-naphthol, the two isomeric sulphuric acid esters of 1:2-dihydroxynaphthalene, 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid and a little 1-naphthyl sulphate.

Attempted preparation of the sulphuric acid esters of (±)-trans-1:2-*dihydro*-1:2-*dihydroxynaphthalene*. Chlorosulphonic acid (0·3 g.) was added carefully to pyridine (5 ml.), followed by (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (0·5 g.). The solution was kept at about 40° for 3 hr., when two layers of liquid formed. The mixture was poured into an ice-cold solution of KOH (1 g.) in water (20 ml.) and the solution was evaporated under reduced pressure until crystals separated. These were collected and extracted with boiling methanol. The methanol was removed under reduced pressure and the solid (120 mg.) recrystallized from ethanol–ether in flat needles, m.p. 164°, which appeared to consist of the potassium salt of (±)-*trans*-1:2-dihydro-2-hydroxy-1-naphthyl sulphate, together with some of the 2-isomer. Because of their lability, attempts to separate the isomers were unsuccessful. On paper chromatograms developed with either solvent 1 or solvent 2 (Table 1) (R_F 0·23 in solvent 1), the crystals formed dark absorbent spots when viewed under ultraviolet light, from which streaked a bright-blue fluorescent compound which, from its colour reactions, appeared to be 1-naphthyl sulphate. 1-Naphthol was also detected. The appearance of the chromatograms suggested that the sulphuric acid esters were decomposing on the chromatograms during development. Further evidence that decomposition was occurring was obtained from the light-absorption curves. A freshly prepared solution of the crystals in water (at pH 6·5) had a maximum at 264 mμ (ε 3900), the expected maximum for a 1:2-dihydronaphthalene type of compound. By the end of 6 hr., this had been replaced by maxima at 272 and 282 mμ and the whole curve was identical with that of potassium 1-naphthyl sulphate. When solutions of the crystals in water were acidified with HCl and examined on paper chromatograms, 1-naphthyl sulphate and a little 1- and 2-naphthol were detected. No 2-naphthyl sulphate was found, but it is not easy to detect small amounts of this ester in the presence of large amounts of 1-naphthyl sulphate. When the ester was heated at 100° with 5N-HCl for 15 min., only 1- and 2-naphthol were detected by paper chromatography and the mother liquors contained inorganic sulphate.

RESULTS

In agreement with Corner & Young (1954), 2- but not 1-naphthol was isolated from the urine of rabbits dosed with naphthalene, although the presence of 1-naphthol was detected on paper chromatograms. The amounts of free *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and the relative proportions of the optically active forms of this compound in the urine of rabbits treated with naphthalene are extremely variable: presumably they depend in part on the methods of collection and storage of urine.

Although 1-hydroxy-2-naphthyl sulphate has not been isolated from the urine of rabbits treated with naphthalene, paper chromatography shows the presence of a compound with the same properties as the synthetic material in the urine of these animals. The compound is also formed when rats are dosed with the isomeric 2-hydroxy-1-naphthyl sulphate, when rabbits, rats and guinea pigs are dosed with (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene and when rats are dosed with (−)-*trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid. 1-Hydroxy-2-naphthyl sulphate couples much more readily with diazo reagents than its isomer and it is presumably this compound which Sato, Yamada, Suzuki, Fukuyama & Yoshikawa (1956) detected by the diazo reaction, in their experiments on the action of rat-liver slices and homogenates on naphthalene and *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and in the urine of rats treated with naphthalene.

The structure of the two forms of *trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid are confirmed by the nature of the decomposition products obtained: mild acid treatment yielded 1-naphthylglucosiduronic acid and a little 2-naphthol,

Table 2. *Ultraviolet absorption of compounds related to* trans-1:2-*dihydro*-1:2-*dihydroxynaphthalene*

Measurements were made in ethanol unless otherwise stated.

Compound	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$
1:2-Dihydronaphthalene*	262	10 230
(±)-*trans*-1:2-Dihydro-1:2-dihydroxynaphthalene (synthetic)†	263	6 830
(−)-*trans*-1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid	264	6 600
(−)-*trans*-1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid (creatinine salt)	262‡	25 400‡
(+)-*trans*-1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid	265	6 900
(−)-Methyl (2-acetoxy-1:2-dihydro-1-naphthyltri-*O*-acetylglucosid)uronate	262	8 200
(+)-Methyl (2-acetoxy-1:2-dihydro-1-naphthyltri-*O*-acetylglucosid)uronate	264	7 800
(−)-Methyl (1-acetoxy-1:2-dihydro-2-naphthyltri-*O*-acetylglucosid)uronate	262	7 900

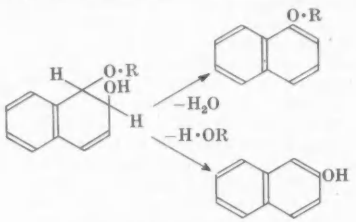
* Morton & de Gouveia (1934). † Beale & Roe (1951). ‡ In water.

strong acid treatment yielded 1- and a little 2-naphthol and hydrolysis with β-glucuronidase yielded either (+)- or (−)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene. 1:2-Dihydro-1-hydroxy-2-naphthylglucosiduronic acid, on the other hand, yielded 1-naphthol (but no detectable 2-naphthylglucosiduronic acid) after both mild and vigorous acid treatment. It is generally found that, with derivatives of 1:2-dihydronaphthalene of this type, treatment with acid yields mainly 1-substituted naphthalene derivatives. The light-absorption curves of the glucosiduronic acids and their derivatives are similar to one another and to that of 1:2-dihydronaphthalene itself (cf. Beale & Roe, 1951) (see Table 2). Although the (−)-form of *trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid readily formed a highly crystalline creatinine salt, the (+)-form failed to do so under all the conditions tried.

The product obtained by the action of chlorosulphonic acid on (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene decomposed spontaneously in aqueous solutions at room temperature in the course of a few hours, immediately with dilute mineral acid and even in the solid state. The breakdown products in each case were 1-naphthyl sulphate, together with a little 1- and 2-naphthol. By analogy with the mild acid decomposition of the corresponding glucosiduronic acids, the original product appears to be a mixture of the two isomeric sulphuric acid esters, the 1-substituted ester breaking down to 1-naphthyl sulphate and 2-naphthol and the 2-substituted ester to 1-naphthol. The sulphuric acid ester has not been detected in the urine of animals treated with naphthalene.

DISCUSSION

In experiments *in vitro* in which naphthalene is incubated with rabbit- or rat-liver microsomal fractions (Mitoma, Posner, Reitz & Udenfriend, 1956; Booth & Boyland, 1958) and with rat-liver slices (Booth, Boyland & Sims, unpublished observations), 1-naphthol or 1-naphthyl sulphate is detected but not the 2-isomers. In animals treated with naphthalene or with *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, free 2-naphthol is present (usually in greater amounts than free 1-naphthol), but 2-naphthyl sulphate or glucosiduronate has not been detected. As Berenbom & Young (1951) have shown that injected 2-naphthol is readily converted into these conjugates in the body, it seems likely that 2-naphthol is not formed by the direct hydroxylation of naphthalene in the way that some at least of the 1-naphthol is formed. Corner & Young (1955) suggested that 2-naphthol, together with 1-naphthyl sulphate and glucosiduronate, arose from the decomposition of the 1-sulphuric acid and glucuronic acid conjugates of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene.



R = SO_3H or $C_6H_9O_6$

Evidence presented in this paper supports this view. Moreover, the 2-glucuronic acid conjugate of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene does not break down to give 2-naphthylglucosiduronate and probably the corresponding sulphuric acid ester does not yield 2-naphthyl sulphate. Although alcohols do not apparently conjugate with sulphuric acid in the body, the formation of such conjugates of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, which are subsequently decomposed in the manner indicated above or dehydrogenated to the sulphuric acid esters of 1:2-dihydroxynaphthalene, would account for the observed pattern of excreted sulphuric acid esters. A similar breakdown of some of the glucuronic acid conjugates of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene would account for the pattern of glucosiduronic acids excreted. In both cases, some at least of the 1-naphthyl conjugates probably arise directly from 1-naphthol. Boyland & Wiltshire (1953) suggested that *trans*-1:2-dihydro-1:2-dihydroxynaphthalene itself [which gives rise to 1- and 2-naphthol with mineral acid (Boyland & Sims, 1953)] might be a precursor of the naphthols, and the 1-naphthylmercapturic acid precursor (Boyland & Sims, 1958) is known to give rise to the naphthols on acidification. In the first case, however, rather drastic conditions are required to effect the breakdown and in the second the formation of the naphthols is accompanied by the formation of 1-naphthylmercapturic acid, which is not usually present in freshly voided urine.

The present work supports the findings of Corner & Young (1955) that the conjugates of 1:2-dihydroxynaphthalene, which are found, together with 2-naphthol and 1-naphthyl sulphate and glucosiduronic acid, in the urine of animals treated with naphthalene are also formed when *trans*-1:2-dihydro-1:2-dihydroxynaphthalene is administered. Sato *et al.* (1956) have found that *trans*-1:2-dihydro-1:2-dihydroxynaphthalene is converted into conjugates of 1:2-dihydroxynaphthalene by the supernatant of rat-liver homogenates and

Fernley & Evans (1958) have shown that naphthalene is converted into 1:2-dihydroxynaphthalene itself by soil pseudomonads. The conjugates of 1:2-dihydroxynaphthalene are not found in the urine of animals treated with 1- or 2-naphthol. It is probable therefore that the conjugates of 1:2-dihydroxynaphthalene found in the urine after the administration of naphthalene arise via *trans*-1:2-dihydro-1:2-dihydroxynaphthalene. This aromatization of non-aromatic compounds is not uncommon. Beer, Dickens & Pearson (1951) have shown for example that hexahydrobenzoic acid is converted into benzoic acid by rabbits. There is no evidence as yet whether it is the free or conjugated dihydrodihydroxy compounds or both which are dehydrogenated.

The metabolism of naphthalene in the rabbit appears to proceed by three main routes: (1) A direct hydroxylation to 1-naphthol followed by conjugation with sulphuric acid or glucuronic acid. (2) A 'perhydroxylation' followed by conjugation and breakdown as outlined above, or dehydrogenation of either the free or conjugated 'perhydroxylated' product to give sulphuric acid and probably glucuronic acid conjugates of 1:2-dihydroxynaphthalene. (3) The reaction of some reactive intermediate with the SH groups of cysteine, glutathione or a protein to give, after hydrolysis and acetylation, the mercapturic acid precursor. The nature of this reactive intermediate is still uncertain, but if it is the epoxide, as suggested by Boyland (1950), then it might be that both routes 2 and 3 start from this same intermediate.

SUMMARY

1. The isolation of the (+)- and (−)-forms of *trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid from the urine of rabbits dosed with naphthalene is described. The (−)-form forms a well-defined creatinine salt.

2. Both the glucosiduronic acids were also isolated in the form of their tetra-acetyl methyl ester derivatives and a third derivative was shown to be that of *trans*-1:2-dihydro-1-hydroxy-2-naphthylglucosiduronic acid.

3. Rabbits convert naphthalene into 1-hydroxy-2-naphthyl sulphate, which is also formed when rabbits, rats and guinea pigs are treated with *trans*-1:2-dihydro-1:2-dihydroxynaphthalene. The ester was synthesized by the persulphate oxidation of 1-naphthol.

4. Possible mechanisms of formation of the above-mentioned compounds in the animal body are discussed.

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Site of Synthesis of the Peptide Component of the Cell Wall of *Bacillus megaterium*

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Gale & Folkes (1953) showed that single radioactive amino acids are still incorporated into the protein fraction of *Staphylococcus aureus* in the presence of growth-inhibitory concentrations of chloramphenicol. More recently, the bulk of this residual incorporation of amino acids has been found to represent synthesis of the hexosamine-peptide polymer which is a major constituent of the bacterial cell wall (Mandelstam & Rogers, 1958; Hancock & Park, 1958). Similar conclusions were reached by Crathorn & Hunter (1958) when studying the chloramphenicol-resistant uptake of [^{14}C]amino acids by *Bacillus megaterium*.

It was also shown that the initial uptake of L-[^{14}C]alanine in *B. megaterium* was located in the cytoplasmic (protoplast) membrane of the cell. It thus seemed likely that the peptides of the cell wall were elaborated at the same site as the mass of the cellular protein (Hunter, Crathorn & Butler, 1957; Butler, Crathorn & Hunter, 1958). More detailed observations on the uptake of alanine into the cell-wall protein are now given together with similar experiments on the incorporation of diaminopimelic acid.

METHODS

Organism. Strain KM of *Bacillus megaterium* was cultured as previously described (Butler *et al.* 1958). For all the experiments detailed here, the organism was grown on the 'C-glucose' medium of McQuillen (1955).

Experiments on the incorporation of [^{14}C]*amino acids by* B. megaterium *in the presence of chloramphenicol.* Bacteria grown overnight were harvested and re-incubated at 30° in fresh 'C-glucose' medium (1 mg./ml.) until they re-entered the logarithmic phase of growth. Chloramphenicol (100 μg./ml. unless otherwise stated) was added and the incubation continued for 15 min. before addition of the appropriate labelled amino acid.

Incubations were terminated by addition of an equal volume of ice-cold aq. 20% NaCl. Lysozyme (0·2 mg./ml.) was added and the bacterial suspension was gently shaken at 30° until the conversion into protoplasts was complete. The protoplasts were separated from the lysozyme digest by sedimentation. Membrane and cytoplasmic fractions were then obtained by adding ice-cold water to the protoplasts and sedimenting the membrane fraction at 10 000–20 000 *g* in the usual way (Butler *et al.* 1958).

Where the whole cell-wall fraction was required, the whole cells were sedimented from the suspension in ice-cold aq. 20% NaCl without any addition of lysozyme. The cells were then disintegrated ultrasonically as described below.

Preparation of cell walls from Bacillus megaterium. In our hands, the preparation of cell walls from *B. megaterium* by shaking with glass beads in the Mickle disintegrator (Salton, 1953) has given less satisfactory results than the method outlined below.

Whole cells (1·65 g.) were suspended in water (5 ml.) and subjected to ultrasonic radiation from an MSE–Mullard tissue disintegrator for five successive 30 min. periods. The temperature was maintained as low as possible throughout the treatment. At the end of each 30 min. period, whole cells were sedimented at 500 *g* and then resuspended for the next ultrasonic treatment. The combined supernatant fractions were centrifuged at 10 000 *g* for 10 min. and the sedimented cell-wall fraction was washed twice with water and twice with 0·06 M-phosphate buffer (KH_2PO_4 brought to pH 7·0 with NaOH); each time the previous sediment was redispersed ultrasonically.

The crude cell-wall preparation obtained (244 mg.) was analysed by acid hydrolysis followed by two-dimensional paper chromatography. The normal cell-wall constituents of *B. megaterium*, namely, alanine, glutamic acid and diaminopimelic acid (Ingram & Salton, 1957), were the principal amino acids present but all the other common amino acids were detectable. The contaminating material could be removed by prolonged digestion with trypsin and ribonuclease in neutral phosphate buffer, but the yield of purified cell wall was then only 106 mg. from 214 mg. of the crude preparation.

Biosynthesis of [^{14}C]*diaminopimelic acid.* A lysine-requiring *Escherichia coli* mutant was grown on the minimal medium of Davis & Mingioli (1950) supplemented with 500 μc of sodium [1-^{14}C]acetate. Other conditions and time of harvesting the bacteria were as described by Work & Denman (1953). [^{14}C]Diaminopimelic acid was isolated from the culture medium by a modification of the procedure of Work & Denman (1953). The culture medium (300 ml.) obtained after harvesting the cells was adjusted to pH 3·0 with conc. HCl and boiled for 5 min. The slight precipitate obtained on cooling was spun off and the clear solution passed through a column of Amberlite 1R 120 (H form; 10·5 cm. × 2 cm.) previously washed until neutral. The column filtrate contained no ninhydrin-positive material. Elution of this column with aq. 2 N-NH_3 soln. removed all the absorbed amino acids in the first 100 ml. After evaporation to dryness the amino acid mixture was taken up in 10 ml. of 1·5 N-HCl and applied to a column of Zeo-Karb 225 (H form; 20 cm. × 2 cm.). The column was eluted with 1·5 N-HCl and 25 ml. fractions were collected automatically. Fractions 17–22 were found to contain

diaminopimelic acid and on concentration yielded diaminopimelic acid dihydrochloride. Conversion into the free amino acid gave diaminopimelic acid (21 mg.) at a specific activity of 0·2 μc/mg. The overall radiochemical yield from sodium [1-^{14}C]acetate was 0·84%.

Paper chromatography and radioautography of hydrolysed protein and peptide fractions. These processes were carried out as described previously (Hunter, Brookes, Crathorn & Butler, 1959).

Materials. Generally labelled L-amino acids (5–9 mc/m-mole) and sodium [1-^{14}C]acetate (2 mc/m-mole) were supplied by The Radiochemical Centre, Amersham, Bucks. [1-^{14}C]Glycine (10·3 μc/mg.) was synthesized in these laboratories. All the labelled compounds were used without any dilution by non-labelled material.

Assays of radioactivity. Protein and other samples were prepared for assay of radioactivity as described previously (Crathorn & Hunter, 1957; Butler *et al.* 1958). End-window counting of solid ^{14}C samples was carried out as before (Crathorn & Hunter, 1957). The specific radioactivities of cell-wall 'protein' fractions were usually calculated by difference from the values obtained on the whole cell, the membrane and the cytoplasmic fractions. It was found that the calculated results agreed with those determined by direct isolation of the cell walls if the cell-wall 'protein' was assumed to account for 30% of the cellular protein, the membrane fraction 25% and the cytoplasmic fraction 45%. Errors due to adsorption, etc., are not important (see Hunter *et al.* 1959).

RESULTS

Effect of chloramphenicol on the incorporation of labelled amino acids into the protein fractions of the cell. In the presence of growth-inhibiting concentrations of chloramphenicol, it was found that the rate of uptake of several labelled amino acids into the protein of the whole cell was reduced to 2–4% of that in its absence. With L-alanine, which occurs in the cell wall, the observed inhibition in the amount of its incorporation into whole-cell protein was only 40–85%. The rate of uptake of glycine, which might perhaps undergo a limited metabolic conversion into a cell-wall constituent, was also inhibited by chloramphenicol to a slightly less extent than most of the other amino acids tested. Typical results are shown in Table 1. A closer examination of the results obtained with L-alanine showed that, although the bulk of the chloramphenicol-resistant incorporation was obviously located in the cell wall, the labelling of the membrane fraction was also inhibited to a much smaller extent than that of the cytoplasmic fraction.

A time study was therefore made of the uptake of L-[^{14}C]alanine into the various protein fractions in the presence of chloramphenicol. It revealed (Fig. 1) that at the earliest times the protein of the cytoplasmic membrane had the highest specific radioactivity. When incubations were prolonged beyond 2 min. the cell-wall protein was more heavily labelled.

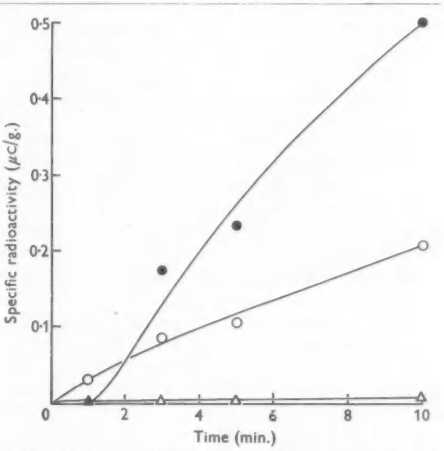

Fig. 1. Uptake of L-[^{14}C]alanine (10 μc) into the protein of the various cellular fractions in the presence of chloramphenicol. Conditions of incubation and sampling were as described in the text. Altogether 1 g. of whole cells was used in the experiment. ●, Cell wall (calc.); ○, cytoplasmic membrane; △, cytoplasm.

Table 1. *Uptake of labelled amino acids into the various cellular-protein fractions in the presence and absence of chloramphenicol*

The whole cells (250 mg. for each treatment) were incubated with or without chloramphenicol as described in the text. A portion (2 μc) of the [^{14}C]amino acid to be examined was added and the incubations were continued for a further 30 min. One-fifth of the suspension was then withdrawn and treated with trichloroacetic acid (to 5%), and membrane and cytoplasm fractions were prepared from the remainder of the suspension as described in the text.

[^{14}C]Amino acid added	Chloramphenicol	Specific radioactivity (μc/g.) of the protein		
		Whole cell	Membranes	Cytoplasm
L-Alanine	–	1·83	1·42	1·44
	+	0·33	0·20	0·02
Glycine	–	0·97	1·02	1·14
	+	0·073	0·059	0·038
L-Valine	–	1·89	1·94	2·54
	+	0·037	0·049	0·060

Supplementary experiments with L-[^{14}C]*alanine.* These were designed to test the hypothesis that the cell-wall peptides were initially synthesized within the cytoplasmic-membrane fraction. In the first place, cells labelled for 20 min. in the presence of chloramphenicol were washed and then 'starved' for 2 hr. by incubating them at 30° in a neutral phosphate buffer containing no labelled substances. They were then harvested and suspended in 'C-glucose' medium before re-incubating at 30°, chloramphenicol being present all the time. Radioactive assays showed that the radioactivity of the cell-wall peptides rose rapidly on re-incubation. However, the specific radioactivity of the membrane protein also rose slowly (Fig. 2).

In another experiment, whole cells were converted into protoplasts and then incubated at 30° for 20 min. with L-[^{14}C]alanine as described elsewhere (Hunter *et al.* 1959), but with the addition of chloramphenicol (0·1 mg./ml.) to the medium. The protoplasts were harvested and the radioactivity of the protein in the medium was determined with the aid of added 'carrier' serum albumin. However, there was at least ten times as much labelled protein in the membrane fraction as in the medium. A similar experiment was carried out with the isolated membrane fraction, again as described elsewhere (Hunter *et al.* 1959), with similar results. It was noteworthy, however, that the uptake of L-[^{14}C]alanine with membrane protein was inhibited to a lesser extent (80%) by chloramphenicol than was L-valine (96%), for instance.

In view of the known inhibitory effect of penicillin on cell-wall synthesis (Park & Strominger, 1957), cells were incubated as usual with L-[^{14}C]-alanine, in the presence of both chloramphenicol and penicillin. The incorporation of L-[^{14}C]alanine into the cell-wall peptides was stopped completely by the combination of antibiotics, but the incorporation of label into the membrane-protein fraction was not affected (Table 2).

To check the possibility that chloramphenicol might act by inhibiting cytoplasmic enzymes responsible for transferring labelled peptides from the membranes to the cytoplasm, membranes labelled in the normal way (Butler *et al.* 1958) with L-[^{14}C]alanine were incubated with cytoplasm treated with chloramphenicol (0·1 mg./ml.). Radioactivity was transferred to the cytoplasmic-protein fraction as usual (Table 3).

Incorporation of [^{14}C]*diaminopimelic acid and* L-[^{14}C]*aspartic acid in the presence of chloramphenicol.* Initial experiments showed that the bio-

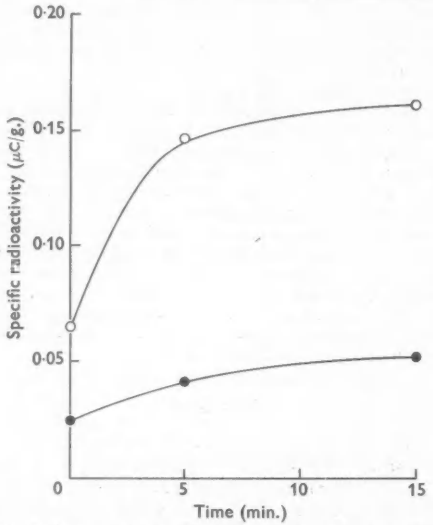

Fig. 2. Increase in specific radioactivity of protein from cell-wall and membrane fractions upon re-incubation of whole cells in 'C-glucose' medium. The cells (750 mg.) were initially labelled with L-[^{14}C]alanine (6 μc) in the presence of chloramphenicol as described in the text. They were then washed and suspended (1 mg./ml.) in 0·1 M-KH_2PO_4 (brought to pH 7·0 with NaOH) and incubated at 30° for 2 hr. in the presence of chloramphenicol (0·1 mg./ml.). They were finally resuspended (1 mg./ml.) in 'C-glucose' medium containing chloramphenicol (0·1 mg./ml.). ○, Cell wall (calc.); ●, cytoplasmic membrane.

Table 2. *Combined effect of penicillin and chloramphenicol on the uptake of* L-[^{14}C]*alanine into the protein fractions of the cell*

Cells (250 mg. for each treatment) were incubated for 15 min. as described in the text in the presence or absence of penicillin (0·5 mg./ml.) and chloramphenicol (0·1 mg./ml.). L-[^{14}C]Alanine (5 μc) was added in each case and the incubations were continued for a further 20 min. before terminating them as described in the text.

Addition	Specific radioactivity of protein (μc/g.)		
	Cell walls (calc.)	Cytoplasmic membrane	Cytoplasm
—	8·4	4·3	4·3
Chloramphenicol	4·1	1·6	0·04
Chloramphenicol + penicillin	0·0	1·9	0·05

Table 3. *Effect of chloramphenicol on the transfer of labelled protein from the membrane fraction to cytoplasm*

The membrane fraction was labelled *in vitro* with L-[^{14}C]alanine, and the transfer experiments were carried out according to Butler, Crathorn & Hunter (1958). Chloramphenicol was used at 1 mg./ml., and both treated and untreated cytoplasm were incubated at 30° for 15 min. before carrying out the transfer.

	Final radioactivity (mμc)		
	Membrane	Cytoplasm	Transfer (%)
Untreated cytoplasm	12·0	3·5	23
Cytoplasm treated with chloramphenicol	8·0	2·7	25

Table 4. *Incorporation of* L-[^{14}C]*lysine,* L-[^{14}C]*aspartic acid and* [^{14}C]*diaminopimelic acid into the whole cellular protein in the presence of chloramphenicol*

Whole cells (150 mg.) were treated with chloramphenicol as described in the text and divided into three parts, each of which was re-incubated with the amino acid concerned (1 μc). Samples were taken at intervals, treated with trichloroacetic acid and the protein was isolated and assayed in the usual way.

Incubation time (min.)	Specific radioactivity (μc/g.) of protein from cells treated with		
	L-[^{14}C]Lysine	L-[^{14}C]Aspartic acid	[^{14}C]Diaminopimelic acid
15	0·40	1·20	0·32
30	0·38	1·62	0·24
60	0·46	1·64	0·32
120	0·50	1·90	0·68
240	0·44	1·82	0·60

synthetic [^{14}C]diaminopimelic acid was not very efficiently incorporated by the whole cell. A comparison of the incorporation of L-[^{14}C]lysine and [^{14}C]aspartic acid, both possible biosynthetic precursors of diaminopimelic acid, with the incorporation of [^{14}C]diaminopimelic acid itself (Table 4) showed that in the presence of chloramphenicol the L-aspartic acid label was utilized much more readily than the ^{14}C of the diaminopimelic acid. This does not mean, of course, that the aspartic acid was incorporated more efficiently by weight than the diaminopimelic acid, which had a specific radioactivity about 100 times less than that of the aspartic acid. However, as [^{14}C]diaminopimelic acid of high specific activity was not available, a closer examination of the incorporation of [^{14}C]-aspartic acid was made.

A time study of the uptake of L-[^{14}C]aspartic acid into the different parts of the cell showed (Fig. 3) that, as with L-[^{14}C]alanine, the incorporation of aspartic acid was practically restricted to the cell-wall and membrane fractions. The cell-wall and membrane protein fractions were further examined by total acid hydrolysis, paper chromatography and radioautography. In all cases the principal radioactive component (about 90%) of the membrane and wall fractions was found to be diaminopimelic acid.

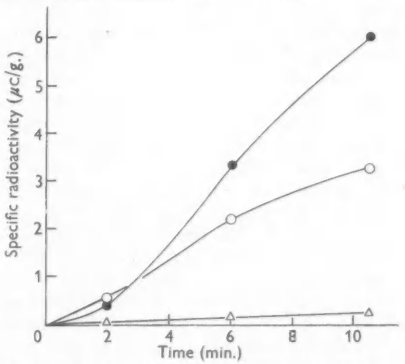

Fig. 3. Uptake of L-[^{14}C]aspartic acid (30 μc) into the protein of the various cellular fractions in the presence of chloramphenicol; 75 mg. of the whole cells was used. Conditions of incubation and the subsequent operations were as described in the text. ●, Cell wall (calc.); ○, cytoplasmic membrane; △, cytoplasm.

DISCUSSION

As more information became available about the composition of bacterial cell walls, it became obvious that the amino acids whose incorporation into the protein of *Staphylococcus aureus* was least

affected by chloramphenicol (Gale & Folkes, 1953) were also important constituents of the staphylococcal cell wall. Our results show that a similar situation exists in *Bacillus megaterium*, where the incorporation of L-alanine, a cell-wall constituent, and of L-aspartic acid, the biochemical precursor of diaminopimelic acid, are less affected by chloramphenicol than is the incorporation of other amino acids. With *S. aureus*, two groups of workers (Mandelstam & Rogers, 1958; Hancock & Park, 1958) showed that the bulk of the chloramphenicol-resistant uptake could indeed be accounted for by the incorporation of amino acids into cell-wall peptides. However, neither group of workers examined the labelling of the cytoplasmic-membrane fraction and the methods used would not have detected even a considerable amount of radioactivity in this fraction. With *B. megaterium*, our time studies of the uptake of L-[^{14}C]alanine into the various cellular fractions show that in the presence of chloramphenicol the protein of the cytoplasmic membrane fraction becomes significantly labelled. In fact, at the shortest times, the membrane protein carries a higher specific radioactivity than the cell-wall peptides. This is reminiscent of the situation with regard to cytoplasmic-protein synthesis (Butler *et al.* 1958), and suggests that the site of synthesis of the cell-wall peptides also lies within the cytoplasmic-membrane fraction.

Further support for this idea was difficult to obtain. Thus cells labelled with L-[^{14}C]alanine in the presence of chloramphenicol were 'starved' in phosphate buffer in the expectation that on reincubation in a full medium, labelled peptides would pass from the membrane fraction into the cell wall. However, the endogenous pool of L-alanine in the cell apparently remained so high that the amount of labelled material in the membrane protein actually continued to rise on reincubation. The specific radioactivity of the cell-wall peptide fraction certainly rose much more rapidly (Fig. 2), but this could be construed as no more than an indication of passage of labelled peptides from membrane to wall.

Nothing definite could be deduced from the experiments with protoplasts or the membrane fraction, although a lower degree of inhibition of the uptake of L-alanine into protein was observed. Only very small amounts of radioactive material passed from these fractions into the medium, which suggested that cell-wall receptors are necessary before labelled precursors are released from the membrane fraction.

Again, the experiments with penicillin were inconclusive. In one respect, however, the information obtained was valuable. It showed that the labelled peptide formed in the membrane fraction in the presence of chloramphenicol could hardly be due to the presence of residual wall mucopeptide not removed by lysozyme. Its formation was not affected by penicillin, which reduced the labelling of the cell-wall fraction itself to zero. Apparently penicillin, even in the high concentration used, does not inhibit the formation within the membrane fraction of precursors of cell-wall peptides. This finding is, however, in conformity with Park's (1958) suggestions on the mode of action of penicillin. He considers it 'probable that penicillin interferes with cell-wall synthesis by preventing the incorporation of the *N*-acetylmuramic acid peptide fragment into the wall'. However, results of our experiments on the transfer of labelled protein *in vitro* from the membrane to cytoplasm poisoned with chloramphenicol do make it unlikely that chloramphenicol has a corresponding action on the passage of peptides or proteins into the cytoplasm.

We decided that to obtain conclusive information it would be necessary to study the uptake of an amino acid present only in the cell-wall peptides. Diaminopimelic acid is apparently absent from the cytoplasmic proteins, but the initial experiments with the biosynthetic [^{14}C]diaminopimelic acid soon revealed that very large quantities of the labelled compound would be required to obtain useful results. However, it is known that L-aspartic acid is an immediate biosynthetic precursor of diaminopimelic acid in some organisms (work summarized and extended by Gilvarg, 1958), and it was soon found that the ^{14}C of L-aspartic acid was efficiently incorporated into the 'protein' fractions of both cell wall and membrane by *B. megaterium*. It is not certain from our experiments that L-aspartic acid is actually used more efficiently by the cell than the diaminopimelic acid, as we have no information about the relative 'pool' sizes of the two amino acids within the cell. But certainly we could obtain much heavier labelling of the protein fractions concerned when using the L-aspartic acid.

Conclusive evidence that aspartic acid was being converted by the cell into diaminopimelic acid was obtained when the proteins of membranes and cell walls, labelled with L-[^{14}C]aspartic acid in the presence of chloramphenicol, were hydrolysed and the products examined by chromatography and radioautography. With both membranes and cell walls the major labelled amino acid was diaminopimelic acid, which was estimated to contain approximately 90 % of the radioactivity present in the hydrolysate. These results seem to give adequate confirmation of the hypothesis that the peptide components of the cell wall of *B. megaterium* are synthesized at sites on or closely associated with the cytoplasmic membrane.

SUMMARY

1. In the presence of growth-inhibitory concentrations of chloramphenicol, the incorporation of most L-[^{14}C]amino acids into the protein fractions of *Bacillus megaterium* is inhibited to the extent of about 96%.

2. The incorporation of L-[^{14}C]alanine into the cellular protein is only 50–85% inhibited by the same concentration of chloramphenicol. The residual incorporation can be accounted for largely by the uptake of ^{14}C into the peptide fraction of the cell wall. However, when the L-[^{14}C]alanine is presented to the cell for less than 2 min., the proteins of the membrane fraction are more highly labelled than are the cell-wall peptides.

3. In the presence of growth-inhibitory concentrations of both penicillin and chloramphenicol, L-[^{14}C]alanine is incorporated into the protein of the cytoplasmic-membrane fraction, but not appreciably into the cytoplasmic protein or the cell-wall peptides.

4. In the presence of chloramphenicol, L-[^{14}C]-aspartic acid is still converted relatively efficiently into the protein or peptide fractions of the cell-wall and cytoplasmic membrane. Radioautographs of these fractions show that the radioactivity is mainly associated with diaminopimelic acid.

5. It is concluded that the peptide components of the cell wall of *B. megaterium* are synthesized at sites on or closely associated with the cytoplasmic membrane.

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Loss of Excitability in Isolated Cerebral Tissues, and its Restoration by Naturally Occurring Materials

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Electrical pulses applied to cerebral tissues initiate large and rapid metabolic changes akin to those associated with increased cerebral activity *in vivo* (McIlwain, 1956*b*, 1959). Though general schemes linking impulse and metabolic response can be proposed, unidentified chemical factors are still involved. Previous attempts to identify such factors have been made by placing the tissue under adverse conditions so that metabolic response was lost, and then making specific additions to the treated tissue with the object of restoring response. Often the adverse conditions led to defects which could not be made good in this way, but by applying electrical pulses to the tissue while it was being incubated in the absence of oxygen or of glucose, some success was obtained (McIlwain & Gore, 1953; McIlwain, 1956*a*). The partial restoration which was achieved was, however, obtained with known metabolites: with malic acid or fumaric acid, adenine or nicotinamide.

With the object of identifying new factors, a fresh beginning has been made by exposing cerebral tissues to other adverse conditions. In the belief that requirement for the metabolites just named was perhaps associated with incubation in the absence of substrates, in the present experiments we have employed methods in which tissue was placed in a normally adequate glucose-containing medium, but was kept in the cold or in the presence of adsorbents or of glycerol. The last-named procedure was adopted by analogy with glycerol treatment of muscle (Szent-Gyorgi, 1949). After such treatments, the tissue's excitability has been examined by placing it in fresh oxygenated glucose media, applying electrical pulses and measuring respiratory and other responses. Some experiments investigated the effects of increased concentrations of potassium salts, which also increase the respiration of normal cerebral tissues.

It proved relatively easy to induce in cerebral tissues a defect of the type desired, by standing them in cold fluid media. In subsequent experiments therefore various materials were added to the depleted tissue with the object of restoring its previous response to stimulating agents.

EXPERIMENTAL

Tissue metabolism

Tissues, media and stimulating agents. Guinea pigs were used unless otherwise stated; they were stunned by a blow on the neck, killed by bleeding and the brain was removed. Rabbits were killed by injection of air into an ear vein; human tissues were obtained at neurosurgery. In each case the cerebral cortex was promptly sliced at 0·35 mm. intervals with razor blade and a guide. Phosphate and bicarbonate media were based on those of Krebs (McIlwain, 1951; Rodnight & McIlwain, 1954); a medium buffered with 25 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) at pH 7·4 was also employed, in which this buffer replaced the phosphate buffer of the phosphate medium; all contained 10 mM-glucose and were initially equilibrated with O_2 or (for bicarbonate media) with O_2+CO_2 (95:5). An atmosphere of O_2 was maintained during respiratory measurements but not when the tissue was in cold medium (see below).

Electrical pulses were obtained and measured with the apparatus of Ayres & McIlwain (1953), and applied to tissues held in silver-grid electrodes in conical manometric vessels E (Ayres & McIlwain, 1953). Condenser pulses of exponential time–voltage relationship were employed, alternating, at 100/sec., peak potential 10 v and time constant 0·4 msec. When KCl was to be added during an experiment, it was placed initially in a side arm of a manometric vessel as an M-KCl soln., in quantity sufficient to give a final concentration of 36 mM-K^+ ions, which was chosen as the minimal concentration giving a maximal or near-maximal increase in respiratory rate.

Measurements. Respiration was measured manometrically by pressure readings each 5 min. in conventional manometric apparatus and in that of Ayres & McIlwain (1953). Formation of lactic acid was determined at the end of manometric experiments by Barker & Summerson's (1941) method. In certain experiments in which tris media had been employed, the tissue's content of inorganic and creatine phosphates was determined after their separation by barium salts by the procedures described by Heald (1954). Data are given in terms of moist wt. of tissue.

In most experiments, respiratory response of the tissue to stimulating agents was determined by measuring respiration during 45 min., applying the stimulating agent (KCl or electrical pulses) and continuing measurements for a further 45 min. Glycolytic response to the stimulating agents was measured by comparing the lactic acid formation by tissues treated as just described with the formation by tissues to which no stimulating agent was applied. Typical experiments involved six tissue slices in separate vessels, some with added substances whose effects were being tested. For electrical stimulation, a specific electrode was used in each vessel. Experiments were planned so that the vessels with added substances in one experiment were without added substances in a duplicate experiment; in this way any variation due to electrode arrangements was randomized (variation was not large: Ayres & McIlwain, 1953). Electrical pulses applied as in the present experiments can cause uptake of O_2 in the presence of certain naturally occurring substances (Lewis & McIlwain, 1954). The materials used in the present experiments were therefore examined for such effects in the absence of tissue. No significant absorption of O_2 occurred on passage of pulses in the presence of blood serum, plasma or extracts of muscle and liver. A slight absorption occurred with pulses in the presence of an extract of brain (6 mg. dry wt./ml.) and correction was made for this in the values recorded in Fig. 3. In the presence of plasma fraction IV-4 (see below) a slight absorption of O_2 took place before pulses were applied. Absorption was equivalent to 1–2 μmoles/g./hr. when referred to an assumed slice weight of 50 mg. This value was sufficiently small to be ignored; it was not increased by pulses.

Exposure of tissue to adverse conditions. Standard conditions were: tissues were cut while wet with the medium to be used in the later part of the experiment, drained from excess of medium on a glass surface, trimmed to weigh about 45 mg., and weighed, and those which were to be exposed were placed in 3·5 ml. of the medium in 25 ml. conical flasks in a tray of ice–water which was put in a cold room. The flasks were loosely stoppered; the atmosphere was air. Other tissues which were prepared at the same time but which were not to be exposed were placed, after weighing, in fluid media in manometric vessels, O_2 was passed and metabolic measurements were made immediately. After the exposed tissues had remained in cold media for periods of 1–17 hr., they were removed with a mounted bent wire and medium was drained from them by drawing them up the side of the flask. The tissues were then placed in manometric vessels in fresh media (those to receive electrical pulses being first placed in the grid electrode), and the media were equilibrated with O_2 and respiratory measurements made.

For some experiments, uncut cerebral hemispheres were placed in screw-capped polythene containers of 30 ml. which contained filter paper moistened with the medium of the experiment, and which were kept at 0°. When slices

Table 1. *Respiration and glycolysis of guinea-pig cerebral cortex after keeping it under various conditions*

All slices weighed 45–50 mg. In Expt. 1 these were placed immediately in 3·5 ml. of phosphate–glucose media in manometric vessels; in Expt. 2 the slices were cut after 12 hr. and then similarly used. In Expts. 3–5, slices cut at zero time were placed in the medium 17 hr. later; those of Expt. 5 had been kept in 3·5 ml. of the same medium, but were removed from it to fresh media for the manometric experiment. For other details, see the Experimental section. Values are given ±S.D. with (in parentheses) the number of observations.

Expt. no.	Conditions of keeping at 0°	Time at 0° (hr.)	Respiration (μmoles of O_2/g./hr.) Ordinary medium	Respiration (μmoles of O_2/g./hr.) With 36 mM-K^+ ions	Lactic acid formation (μmoles/g./expt.) Ordinary medium	Lactic acid formation (μmoles/g./expt.) With 36 mM-K^+ ions
1	Control	0	59±4 (9)	101±9 (7)	28±5 (6)	46±6 (4)
2	As whole hemisphere	12	61±6 (3)	97±13 (3)	31±4 (3)	41±7 (3)
3	As slices, cut without medium	17	73±9 (5)	90±17 (5)	—	—
4	As slices, cut in medium and with approx. 50 μl. of medium adhering	17	44±9 (6)	52±6 (5)	—	—
5	As slices, cut in medium and with 3·5 ml. of medium	12	47±4 (8)	52±6 (4)	30 (2)	35 (3)

were to be kept without medium, they were cut without contact with medium and weighed on a wire hook, which was subsequently suspended from the stopper of a 25 ml. conical flask above a small amount of medium which it contained; the flasks were kept at 0°.

Materials examined with exposed tissues

Preparations from blood. Human, guinea-pig and ox sera were prepared from whole blood allowed to clot at 37°. Before use in manometric experiments, CO_2 was removed as described by Warren (1944) and the pH readjusted to 7·4. Dried human serum was also employed; dialysis of the dried serum after reconstitution was carried out at 0–3° in cellophan tubing which was moved mechanically in 4 vol. of water, or other fluid medium, renewed at frequent intervals. The lactic acid of the serum was determined and found to fall from 5·0 to 0·2 mM in 12 hr. dialysis.

Acetone-precipitation of dialysed serum was carried out by adding about 50 ml. of the serum to 4 vol. of acetone at 0° with constant stirring. The mixture was centrifuged and the precipitate washed with further small quantities of acetone and dried in a vacuum desiccator containing paraffin wax. In one instance the precipitate was redissolved in water (6 g./100 ml.) and reprecipitated in the same way. Acetone was removed from the soluble fractions by vacuum-distillation and the residue freeze-dried. Extracts were also prepared from the dialysed serum by heating at pH 5·3 according to Hunter (1957). Chloroform extraction of serum samples or products was by repeated mechanical shaking with 2 vol. of $CHCl_3$.

By courtesy of Mr D. Purdie of Armour Laboratories, Eastbourne, we were supplied with ox-plasma fractions designated I, II, III-1, III-2, IV-1, IV-4 and V by Cohn *et al.* (1946), and prepared by ethanol precipitation at controlled pH. Albumin from fraction V was also obtained from L. Light and Co. Ltd.

Tissue extracts and other materials. Cerebral tissues were extracted in the cold as follows. The brain of a guinea pig or rabbit was removed rapidly and cut with a scalpel to blocks about 3 mm. thick; these were chopped in the machine of McIlwain & Buddle (1953) to prisms about 2 mm. × 0·2 mm. × 0·1 mm. These were suspended in 4 vol. of ice-cold medium buffered with tris and without glucose for (*a*) 0·5 hr. and (*b*) 17 hr. The supernatant from centrifuging (1500 *g*, 10 min.) was used. A third type of extract (*c*) was made in a blender (2 min.) from the brain and 4 vol. of 0·9% NaCl. After standing 40 min. at 0° the mixture was centrifuged at 30 000 *g* for 10 min. at 0°.

Hot extracts were made from brain and from liver by cutting the organs with scissors to approx. 0·5 cm. cubes and dropping these into 4 vol. of water boiling in a beaker, so that the water continued to boil for 3 min. The mixture was then centrifuged at 1500 *g* for 15 min. We are grateful to Dr A. E. Bender (Bovril Ltd.) for supplying us with a sample of commercial meat extract and also of its desalted dialysate, both prepared as described by Wood & Bender (1957). Polyvinylpyrrolidone was kindly provided by May and Baker Ltd.; Nelson's Photographic Gelatin was used.

RESULTS

Conditions for obtaining loss of metabolic response to stimulating agents

A block of cerebral tissue a few grams in weight may be kept in a moist atmosphere at about 0° for at least 10 hr. without marked effect on the rates of respiration which are subsequently shown when it is sliced and incubated in glucose-containing media. Moreover, respiration is increased in nearly normal fashion when electrical pulses are applied to the tissue (McIlwain, Ayres & Forda, 1952). In the present experiments, similar stability has been shown in the tissue's glycolysis and in its respiratory and glycolytic responses to increased concentration of potassium salts. Thus increase in potassium salts from 6 to 36 mM, which increased the respiration of normal tissues by some 70%, increased the respiration of those kept at 0° for 12 hr. by about 60% (Table 1). The stability also extended to tissue sliced before it was kept at 0°, provided that the slicing was carried out in the absence of fluid media. Much greater loss of response to stimulation was, however, found when

the sliced tissue was kept in contact with glucose-containing media for comparable times. Respiration fell and after 17 hr. showed little response to potassium salts; glycolysis changed little but glycolytic response fell.

Keeping in fluid media. It appeared therefore that tissue might be altered in the way desired by keeping slices of cerebral cortex in glucose-containing media at 0°, and this was therefore done under a variety of conditions. The volume of medium with which tissue was kept could be altered considerably and still afford a similar fall in respiration. A 50 mg. slice moistened with only about 50 μl. of medium gave an 18% respiratory response under conditions in which one placed in 3·5 ml. of medium increased respiration by 11% (Table 1). The change in 3·5 ml. of medium was progressive, as is shown in Fig. 1, and the fall in the stimulated rate of metabolism was, throughout, greater than those occurring in the absence of stimulating agent.

Loss of response was examined also in glucose media buffered with tris. This was done because the phosphate concentration in phosphate-buffered media is much greater than in the tissue's normal environment, and makes difficult the determination of tissue phosphate (see below). Loss of response in tris-buffered media was found more rapid than in media with phosphate buffers. The tissue's response to potassium salts and to electrical pulses changed in comparable fashions (Fig. 2). Changes in the response of slices of the cerebral cortex from other animal species were also studied after they were kept at 0° for 5 hr. in tris-buffered media. Slices from the rabbit, which normally increased in respiration by 86% with applied pulses, after keeping increased by 43%; with those from man the increase before keeping was 71% and after keeping was only 12%.

Change in lactic acid formation on keeping tissues at 0° was studied, and results with guinea-pig tissues are included in Table 1 and in Figs. 1 and 2. Lactic acid formation in the absence of stimulating agents was relatively stable over the 17 hr. studied. Formation in the presence of electrical pulses or of 36 mM-KCl diminished, but to a lesser degree than did the tissue's respiration.

Media with added substances. In an attempt to obtain more rapid loss of the tissue's response to stimulating agents, adsorbents were added to the media in which the tissues were being exposed. This

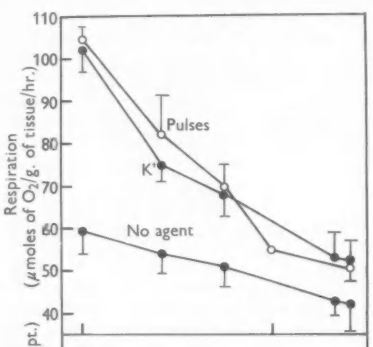

Fig. 1. Change in respiration and in formation of lactic acid on keeping slices of guinea-pig cerebral cortex in phosphate–glucose media at 0°. After being kept for the period indicated by the abscissae, slices were placed in fresh glucose–phosphate media and incubated at 37° for the determinations recorded. Points give mean values of four to seven determinations (S.D. given by vertical lines). ●, Unstimulated tissues and those with 36 mM-potassium salts; ○, tissues with electrical pulses.

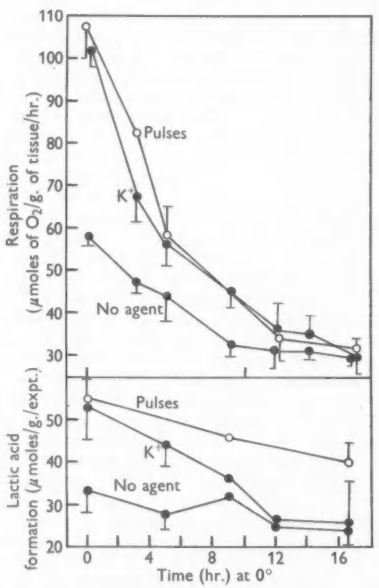

Fig. 2. Change in respiration and in formation of lactic acid on keeping slices of guinea-pig brain in tris–glucose media at 0°. Other details are as given in Fig. 1.

Table 2. *Exposure of guinea-pig cerebral cortex to adsorbents and glycerol*

Adsorbents were first treated with several volumes of medium for about 1 hr. until no further change in pH occurred, before adding them to fresh medium with the tissue. This procedure was adopted after several adsorbents had been found to alter the pH and Ca^{2+} ion content of the medium. The following adsorbents were examined under the conditions of Expt. 2, and induced no significant change in respiration and lactic acid formation: cellulose powder, silica, decolorizing charcoal and calcium phosphate. Values give the mean result, ±S.D. where applicable, of the number of experiments indicated in parentheses.

Expt. no.	Conditions of exposure	Respiration (μmoles of O_2/g./hr.) Ordinary medium	Respiration (μmoles of O_2/g./hr.) With 36 mM-K^+ ions	Lactic acid formation (μmoles/g./expt.) Ordinary medium	Lactic acid formation (μmoles/g./expt.) With 36 mM-K^+ ions
1	At 0°; 1 hr.	55±3 (15)	88±10 (8)	30±5 (7)	50±7 (7)
2	As 1; with alumina, 0·3 g.	48±8 (12)	83±11 (16)	28±6 (6)	49±7 (4)
3	As 1; with 20% (v/v) glycerol	57±3 (4)	95 (2)	30 (2)	50 (2)
4	At 37°; 1 hr.	56 (2)	78 (2)	33 (2)	48 (2)
5	At 37°; 1 hr. with 20% (v/v) glycerol	58±6 (6)	77±8 (6)	34 (2)	54 (2)

had remarkably little effect on the loss of excitability (Table 2). Also, the presence of 20% (v/v) glycerol during depletion was without influence on the process at 0°. At 37°, loss occurred in respiratory response to KCl after 1 hr., but was again uninfluenced by glycerol (Table 2). These conditions induced relatively little change in glycolysis or in the glycolytic response to increased concentrations of KCl.

Tissues were also kept in media which contained blood serum, and were then transferred to ordinary media for metabolic experiments. In this way it was shown that loss of response to KCl in guinea-pig serum at 0° was the same as that occurring in bicarbonate-containing media; under both conditions respiratory rates of 46–47 μmoles of O_2/g./hr. after 17 hr. were unchanged when KCl (final concn. 36 mM) was added. The course of loss of response in bicarbonate medium was similar to that occurring in phosphate media and is shown in Fig. 1. Similar results were obtained after keeping the tissue in human cerebrospinal fluid. Loss of response in a tris-buffered medium with only 1·3 mM-KCl was similar to that occurring in the normal tris medium, which was 6·2 mM in potassium salts. The addition to tris media of mM-creatine, -adenosine and -guanosine together did not prevent the diminution of respiration or respiratory response.

Attempted restoration of response to stimulating agents

Loss of metabolic response to stimulating agents is seen to be gradual. In attempts to restore the response by addition of materials, a few sets of conditions were chosen for the initial loss of response (Table 3). These were: conditions *A* and *B*, after 5 and 17 hr. in phosphate medium, when respiratory response was lowered from its normal 80% to about 45 and 15% respectively; and conditions *C* and *D*, after similar exposure in tris media, when loss was more severe. Substances examined for their ability to restore metabolic response were conveniently incorporated with the media of the subsequent experiments, in which metabolic responses by the tissues were examined. The added substances were thus (see Experimental section) incubated with the exposed tissue at 37° for 45 min. before application of the stimulating agent. The rates of respiration before and after stimulation were the data mainly used to judge the restoration of response caused by the added substance.

Table 3 shows that partial restoration of respiratory response has been achieved with added substances. After exposure for 5 hr. in phosphate media, dilutions of blood sera from guinea pig and from man increased respiration in the absence of stimulating agent and to a rather greater extent after stimulation. In an attempt to obtain greater differentiation between effects on stimulated and unstimulated respiration, tissue was examined after exposure for 17 hr. in phosphate media. Here again restoration of respiratory response was shown, but all increases were relatively small. They were smaller still after the tissue had been standing in tris media for 17 hr. The most suitable conditions for displaying recovery of response were conditions *C* of Table 3: keeping the tissue for 5 hr. in tris medium. Serum added under these conditions is seen to increase response both to electrical pulses and KCl.

These test systems showed that the ability of blood serum to restore respiratory response was not shared by a number of known substances (Table 4). Certain of these depressed the tissue's responses. In appraising the actions of the compounds and also of the preparations from natural sources described below it was especially necessary to be aware of any interactions, extraneous from the

present point of view, between the extract and the stimulating agents, and examination of this is described in the Experimental section. The substances examined (Table 4) are seen to include those previously found to have some ability to restore differently depleted cerebral tissues, and also a number of coenzymes, vitamins and intermediary metabolites. None was found effective in the present system. Also, as it was considered that the activity of blood serum might be related to its colloid properties, polyvinylpyrrolidone and gelatin were examined but were ineffective.

Quantitative comparison and fractionation of natural materials. The preceding results showed that to identify a substance which restored respiratory response, fractionation of naturally occurring materials was likely to be required. For this purpose a quantitative assay was desirable, and Fig. 3 illustrates that which was adopted. Respiratory response of the exposed tissues to

Table 3. *Loss and partial restoration of respiratory response*

The responses recorded were to electrical pulses and to KCl, applied as described in the Experimental section. The control experiments, without added materials, were carried out under the conditions of Figs. 1 and 2. When materials (derived from guinea pigs) were added, they were incorporated into the fresh medium of the second part of the experiment, during which respiration was measured. Respiration values are means ±S.D. with the number of experiments in parentheses.

	Conditions of keeping at 0°		Materials added and amount (mg. dry wt./ml. of medium)	Respiration (μmoles of O_2/g./hr.)			Mean increase in respiration caused by stimulating agent (μmoles of O_2/g./hr.)		(%)	
	Medium	Time (hr.)		No stimulation	With pulses	With K^+ ions	With pulses	With K^+ ions	With pulses	With K^+ ions
A	Phosphate	5	None	50±5 (6)	68±7 (4)	65±4 (3)	18	15	37	30
			Serum, 28	62±6 (3)	100±9 (3)	—	38	—	61	—
B	Phosphate	17	None	43±6 (15)	47±7 (3)	49±6 (5)	4	6	10	12
			Serum, 8·5	40±4 (3)	—	50 (2)	10	—	25	—
C	Tris	5	None	42±5 (15)	53±8 (10)	55±8 (5)	11	13	26	31
			Brain extract (*a*), 5·85	45 (2)	65 (2)	—	20	—	38	—
			Serum, 28	50±6 (15)	80±11 (15)	84±4 (5)	30	34	60	68
D	Tris	17	None	39±5 (15)	39±6 (5)	40±6 (10)	0	0	0	0
			Brain extract (*b*), 4·8	42 (3)	—	42 (2)	—	0	—	0
			Serum, 28	45 (2)	—	52 (2)	—	7	—	15

Table 4. *Materials examined for ability to restore respiratory response to cerebral cortex after the tissue had been kept at 0°*

Preparation of the tissue and examination of its response were carried out according to the conditions of Table 3 (*B*, *C* and *D*). The compounds examined under conditions *D* were employed individually as well as in the groups named, and gave the same result.

Materials examined (mM except when otherwise indicated)	Conditions of keeping at 0° (Table 3)	Results
Fumaric acid: 10, 20; glutamic acid, 10; glutamine, 2; succinic acid, 10, 40; nicotinamide: 2, 4, 20; thiamine, 1·5, 3; inositol, 0·5; ascorbic acid, 1; oxidized and reduced glutathione, 0·1; cozymase, 0·1; adenosine 5-phosphate: 1, 10; adenosine triphosphate: 1, 10	*B*	Response 15% or less with or without the added compounds
Fumaric acid, 5; glutamic acid, 5; oxidized glutathione, 0·1; cytidine, 1; γ-aminobutyric acid, 10; polyvinylpyrrolidone, 1·25%, 2·5%; gelatin, 2·5%	*C*	Response 28% or less with or without the added compounds
Muscle extract, 0·1–0·5%; desalted dialysate of muscle extract, 0·5–2%	*C*	Response 24% with or without added substances
Glutamine, 2; *group i*: glutamic acid, 2 and fumaric acid, 2; *group ii*: thiamine, 1, nicotinamide, 1, creatine, 1, adenosine triphosphate, 1; *group ii* plus fumaric acid, 2; *group ii* plus glutamic acid, 2; *group ii* plus *group i*. Hot extracts of rabbit and of guinea-pig brains (approx. 5 mg. of solids/ml.)	*D*	No response without additions; response 12% or less with added compounds

stimulating agents was expressed as a percentage of their respiration without stimulation and plotted against the quantity of added material.

On this basis, blood serum afforded the greatest restoration of response (Fig. 3, Table 5). Low concentrations of extracts from cerebral tissues or liver had activity equal to or greater than those of corresponding weights of serum, but in greater concentrations the liver and brain were inhibitory. The desalted dialysate of meat extract, described in the Experimental section, was without effect on the exposed tissue when included at 10 mg./ml. of medium. Many constituents of this dialysate have been identified (Wood & Bender, 1957). The meat extract before treatment contained too much salt for testing in the present system. Of the cerebral extracts, (*a*) and (*b*) (see Experimental section) were made by a process similar to that in which the tissue was kept in fluid and by which the test tissue lost its response; and at levels of about 6 mg. of solids/ml. they increased respiratory response from some 25 to 38 % under conditions *C* of Table 3. The extracts produced by blending the tissue greatly increased the respiration of normal as well as of depleted tissue, but had little effect in making good the respiratory response. After a few attempts to

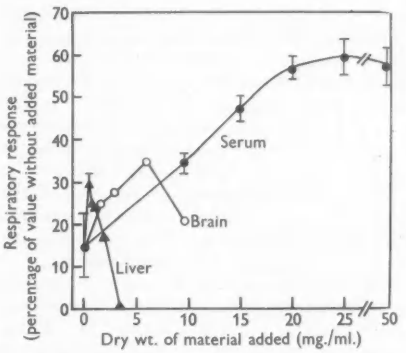

Fig. 3. Effects of various concentrations of tissue preparations in restoring the respiratory response of guinea-pig cerebral cortex previously exposed at 0°. The sliced cortex was exposed under conditions *C*, Table 3, and respiratory response to electrical pulses was measured in tris–glucose media. Response is expressed as the mean percentage increase in respiration caused by the pulses, as in the last columns of Table 3. Serum was reconstituted from dried human serum; tissue extracts were prepared by method *c* (see Experimental section). ▲, Tissue with liver extract; ○, tissue with brain extract; ●, tissue with serum.

Table 5. *Action of blood sera and preparations therefrom on the respiratory response of cerebral tissues previously kept at 0°*

Guinea-pig cerebral cortex was kept at 0° and examined under the conditions *C* of Table 3. Values are followed by S.D. and, in parentheses, the number of observations. The preparations were examined also for effect on the respiratory response of normal tissues (not kept at 0°), and were without action [mean response: 90 % ± 9 (16) in the absence of sera, and 82 % ± 11 (8) with sera].

Material added	Dry wt. (mg./ml.)	Tissue respiration: Rates (μmoles of O_2/g./hr.) Without pulses	With pulses	Increase (%)
None	—	44·5 ± 5 (39)	53 ± 7 (53)	20
Human serum	6	50	59	18
	12	50 ± 4 (4)	60 ± 5 (4)	20
	18·8	48 ± 4 (3)	65·5 ± 6 (3)	37
	24	61 ± 5 (3)	97 ± 7 (3)	59
	47	57·5 ± 4 (3)	82 ± 5 (3)	43
Ox serum	6	50 ± 3 (3)	60 ± 4 (3)	20
	12	51 ± 6 (3)	68 ± 7 (3)	33
	24	56 ± 5 (4)	88·5 ± 7 (4)	58
Guinea-pig serum	10	48 ± 6 (4)	68·5 ± 8 (4)	43
	20	50 ± 5 (4)	89 ± 10 (4)	78
Human serum, reconstituted, before dialysis	5	42·5 ± 5 (8)	49 ± 6 (12)	15
	10	47·8 ± 4·5 (10)	70 ± 5 (14)	44
	15	49 ± 6 (5)	75 ± 7 (5)	53
	20	50 ± 7 (10)	80 ± 8 (10)	60
	30	50 ± 6 (4)	75 ± 8 (4)	50
Human serum, reconstituted, dialysed	5	43 ± 6 (5)	55 ± 6 (6)	28
	10	47 ± 7 (10)	69 ± 8 (10)	47
	15	48 ± 6 (10)	73 ± 7 (10)	52
	20	50 ± 5 (8)	84 ± 8 (10)	68
Human serum, reconstituted, dialysed and acetone-precipitated	0	44 ± 5·8 (17)	51·3 ± 9 (13)	16
	5	39 ± 2·0 (5)	52 ± 4·8 (5)	34
	10	36 (2)	51 (2)	42
	15	39 ± 8 (17)	61 ± 4·8 (15)	57
	20	39 ± 3·2 (4)	64·5 ± 8 (5)	65

separate possible stimulating and inhibiting agents from brain and liver had been unsuccessful, blood serum was examined in more detail.

Activities of human, ox and guinea-pig sera were similar. After dialysis of human serum for periods up to 18 hr., the diffusible material, concentrated by evaporation, was found relatively inactive in restoring the tissue's response. Activity was retained, with little loss, in the non-diffusible fraction (Table 5). From the dialysed solutions it could be precipitated with acetone. Sera from different sources were found similarly active, and further information was sought by examining blood-protein fractions available commercially. These (Table 6; see Experimental section) were derived from ox plasma by the ethanol-fractionation of Cohn *et al.* (1946). Fraction IV-4 was found markedly more active than the others. Its activity was partly lost on heating (Table 6).

Tissue phosphates after loss and restoration of response

Of the many chemical characteristics of the tissue which could be relevant to the loss and restoration of its excitability, the levels of inorganic phosphate and creatine phosphate have been studied first. The brain *in vivo* contains about 3 μmoles/g. of each of these phosphates under normal conditions (see McIlwain, 1959); in tissue removed for the metabolic experiments, but before other treatment, phosphocreatine is seen to be largely lost and much inorganic phosphate formed (Table 7). After incubation in glucose media, inorganic phosphate was restored to its normal level and a large part of the phosphocreatine resynthesized. During the exposure of 5 hr. at 0° in tris–glucose medium, which was adopted for causing loss of response, some resynthesis of phosphocreatine and loss of inorganic phosphate also occurred (Expt. 2, Table 7). These processes were taken further on incubating the tissue after exposure, but neither proceeded as far as in the tissue which had not been kept at 0°. The serum which permitted further respiratory response also caused further resynthesis of phosphocreatine. Moreover, this effect of serum on the phosphates of the unresponsive tissue was the reverse of its effect on tissue incubated without having been kept at 0°. Here the inclusion of serum led to lower phosphocreatine and higher inorganic phosphate than were found in the same media lacking serum. (Of serum constituents, glutamic acid and glutamine have this effect: McIlwain, 1952.) These changes were such that, after incubation in tris–glucose media with serum, the phosphates reached similar levels whether or not the tissue had been kept at 0°.

Table 6. *Ox-plasma fractions and the respiratory response of tissues kept at* 0°

Guinea-pig cerebral cortex was kept at 0° and examined under the conditions *C* of Table 3. Values are from duplicate determinations except when followed by S.D. and, in parentheses, the number of observations.

Plasma fraction added	Dry wt. (mg./ml. of medium)	Respiration (μmoles of O_2/g./hr.) Without pulses	With pulses	Increase in respiration (%)
None	0	46±4 (13)	54±6 (9)	17
I	7·5	58	67	15
	15	57	55	0
II	7·5	42	54	28
	15	43±4 (5)	50·5±3 (5)	17
	30	46±5 (7)	60·5±7 (5)	30
III-1	10	36	46	29
	20	45	49	9
III-2	10	47	59	26
	20	46	59	27
IV-1	5	44±3 (4)	59±4 (4)	34±7
	10	54±6 (5)	81±7 (5)	50±4
	20	49±5 (5)	68±4 (5)	39±13
IV-4	5	51±7 (6)	78±8 (3)	52±9
	10	45±5·4 (6)	76±8 (5)	67±4
	20	43·5±5 (5)	70±10 (4)	62·5±14
IV-4, after 2 min. at 100°	10	52±6 (7)	66±8 (6)	30
V	10	45±4 (3)	66±6 (3)	46
	20	54±5 (3)	74±3 (3)	38
Albumin	5	43±4 (4)	55±5 (4)	28
	10	45±3 (8)	58±7 (7)	29
	30	44±4 (3)	59±6 (3)	34
	60	48±5 (3)	61±6 (3)	27

Table 7. *Inorganic phosphate and phosphocreatine in treated tissues*

Guinea-pig cerebral cortex was prepared at room temperature. Incubation was for 45 min. in O_2 in tris–glucose media at 37°. Reconstituted human serum was added where indicated, at a concentration giving 20 mg. dry wt./ml. Extraction of the tissue was done by grinding in 10% (w/v) trichloroacetic acid. Depletion was under conditions *C*, Table 3. Values are followed by S.D. and, in parentheses, the number of observations.

Group of Expts.	Treatment of tissue before extraction	Phosphocreatine (μmoles/g.)	Inorganic phosphate (μmoles/g.)
1	None	0·15±0·01 (3)	7·2±1·0 (3)
1	Incubated in glucose medium	1·64±0·15 (6)	3·05±0·36 (6)
1	Incubated with serum addition	1·06±0·05 (4)	3·15±0·7 (4)
2	Kept at 0°	0·43±0·02 (4)	4·78±0·58 (4)
2	Kept at 0°, incubated in glucose medium	0·85±0·07 (7)	4·03±0·24 (7)
2	Kept at 0°, incubated with serum addition	1·16±0·14 (6)	3·86±0·30 (6)
3	Kept at 0°, incubated in glucose medium	1·05±0·2 (4)*	4·95±1·5 (4)
3	Kept at 0°, incubated with plasma fraction IV-4 (5 mg./ml.)	1·35 (2)	3·4 (2)
3	Kept at 0°, incubated with plasma fraction IV-4 (10 mg./ml.)	1·55±0·3 (5)*	4·24±1·4 (5)

* In Expts. 3, comparison of the mean of the individual differences (in each experiment) caused by the addition of the plasma fraction, with the mean value for the slices not receiving the addition, indicated the effect of the addition on phosphocreatine to be significant with $P < 0·01$. The effect on inorganic phosphate was not significant.

Moreover, the serum fraction IV-4, possessing greatest ability to restore the tissue's respiratory response, was also found active in permitting resynthesis of phosphocreatine (Expt. 3, Table 7).

DISCUSSION

Under the conditions used in the present study, cerebral tissues have suffered greater loss in their respiratory response to electrical excitation than in their resting respiration, and a specific but unidentified material has been found of which a small quantity can restore response to excitation. It is relevant to consider possible relationships between the restorative material and the change undergone by the tissue on losing its ability to respond to excitation.

(1) Neural tissues excized and placed in media lose a variety of substances, some of which appear in the medium (Winterstein, 1929; McIlwain, 1958). In the present experiments, keeping the tissue in medium (at 0° for 5–17 hr.) is an essential part of the procedure causing loss of response, for tissue kept at the same temperature for the same time but without medium did not suffer loss of excitability. Loss occurred more slowly in a phosphate-buffered medium (30 mM-phosphate) than in a similar medium buffered with tris (2 mM-phosphate), and is seen to involve changes in tissue phosphates.

(2) Nevertheless, material which appeared in the medium when cerebral tissues were suspended in it did not restore excitability. Thus the medium does not appear to be acting simply as a receiver of essential substances diffusing from the tissue; or, if it is so acting, the substances lost are not in a form in which they are readily re-assimilated by the tissue, for the defect induced in the tissue can be made good when other naturally occurring materials are added to the medium in which tissue is respiring.

(3) The material most successful in restoring tissue excitability comprises some 6% of the total proteins of blood plasma (Cohn *et al.* 1946). Its ability to restore the tissue does not receive a simple explanation in terms of its major protein constituents. The fraction IV-4 consists largely of α- and β-globulin. Fraction V, precipitated subsequently and less active in restoring excitability, contains much less of these globulins; but they are contained in fractions II and III, which are precipitated before fraction IV and which are relatively inactive in the test system we have used.

(4) As the metabolic response of cerebral tissues to excitation is greatly affected by the ionic composition of the tissue and of suspending media, it is relevant to note that Solomon, Lionetti & Curran (1956) have offered evidence for a cation-carrying substance in blood, and that Creese, D'Silva & Northover (1958) found that a non-diffusible fraction of blood serum aided the maintenance of the sodium content of isolated diaphragm at its normal (low) values. Also, Lindan, Quastel & Sved (1957) found serum fractions effective in antagonizing an inhibitory effect of chlorpromazine on cerebral tissues, and chlorpromazine was found by

McIlwain & Greengard (1957) to inhibit the metabolic response of cerebral tissues to electrical pulses. Lipids or lipoproteins are suspected of being involved in some of these actions, and the material is being further characterized.

SUMMARY

1. Slices of guinea-pig cerebral cortex have been exposed to a variety of adverse conditions with the object of diminishing their respiratory response to electrical stimulation, while maintaining relatively normal respiratory rates in the absence of stimulation.

2. The greatest differential effect occurred on exposure in tris-buffered glucose-containing media at 0° for 5 hr. These conditions lowered also the tissue's glycolytic response to electrical pulses and its respiratory response to high concentrations of potassium salts.

3. Restoration of the exposed tissue's respiratory response to electrical pulses was sought by adding a variety of substances to media in which the tissue was incubated. Additions included known metabolites, blood sera and extracts of brain, muscle and liver.

4. Blood sera from several animal species were the most active of these materials in restoring respiratory response. Dialysis and solvent precipitation suggested that the activity was associated with the serum proteins. Examination of plasma fractions obtained by the method of ethanol precipitation of Cohn *et al.* (1946) showed the most active fraction to be IV-4, which contains α- and β-globulin.

5. The inorganic phosphate and phosphocreatine of cerebral tissues have been determined after the chosen conditions of exposure and after incubation with blood-protein preparations, and relationships between the exposed tissue and restorative agents have been discussed.

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Studies on the Biosynthesis of Cholesterol

9. FORMATION OF PHOSPHORYLATED DERIVATIVES OF MEVALONIC ACID IN LIVER-ENZYME PREPARATIONS*

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After the discovery by Tavormina, Gibbs & Huff (1956) that mevalonic acid (3:5-dihydroxy-3-methylpentanoic acid) is efficiently converted into cholesterol by rat-liver homogenates, and after the demonstration that this acid furnishes the fundamental building unit in the synthesis of squalene (Cornforth, Cornforth, Popják & Gore, 1957, 1958; Dituri, Gurin & Rabinowitz, 1957) and hence of cholesterol (Isler, Rüegg, Würsch, Gey & Pletscher, 1957), we studied the enzymic transformations of mevalonic acid in liver preparations. It has been

* Part 8: Gosselin & Popják (1959).

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found that a combination of soluble enzymes and microsomes from rat liver catalysed the synthesis of squalene and of cholesterol from mevalonic acid in the presence of adenosine triphosphate, reduced diphosphopyridine nucleotide, triphosphopyridine nucleotide, Mg^{2+} ions, nicotinamide, oxygen and a reducing substance such as cysteine, glutathione or ascorbic acid (Popják, Gosselin, Gore & Gould, 1958). In this paper the isolation from liver-enzyme preparations of two phosphorylated derivatives of mevalonic acid are reported; both substances are intermediates in sterol biosynthesis. The results have been reported briefly at a Ciba Foundation Symposium in May 1958 (Popják, 1959).

METHODS AND MATERIALS

Preparation of liver enzymes. Soluble enzymes and microsomes from rat liver were prepared as previously described (Popják *et al.* 1958). The complete enzyme system, which synthesizes both squalene and cholesterol from mevalonic acid, contained (in a volume of 4 ml.): dialysed soluble enzymes (30–50 mg. of protein), suspension of microsomes (0·2 ml.), adenosine triphosphate (ATP; 10–15 μmoles), reduced diphosphopyridine nucleotide (DPNH; 1 μmole), triphosphopyridine nucleotide (TPN; 0·5 μmole), ascorbic acid (30 μmoles), DL-[2-^{14}C]mevalonic acid (0·5–2·0 μmoles), potassium phosphate buffer (0·1 M, pH 7·5), $MgCl_2$ (4 mM) and nicotinamide (30 mM).

Coenzymes. All the coenzymes used were commercial preparations; their sources were given in an earlier paper (Popják *et al.* 1958).

[^{14}C]*Mevalonic acid.* DL-[2-^{14}C]Mevalonic acid lactone and DL-[1-^{14}C]mevalonic acid lactone were synthesized by Dr Rita Cornforth according to the method of Cornforth *et al.* (1958). Solutions of the lactone (0·1 M) were made in 0·1 M-potassium hydrogen carbonate and were incubated for 30 min. at 37° before use; this treatment caused the opening of the lactone ring and gave the potassium salt of mevalonic acid. Mevalonic acid preparations with specific activities of 0·04 and 0·2 μc of ^{14}C/μmole have been used. The efficiency of our instrument for assaying ^{14}C in infinitely thin samples is about 6%.

Chromatography. For chromatography on Whatman no. 1 paper a variety of solvent systems was used; *iso*-butyric acid–aq. NH_3 soln.–water (66:3:30, by vol.) was found the most convenient and results obtained with this system alone will be shown. Photographic prints with ultraviolet light (Markham & Smith, 1949) were made of the paper strips, which were also scanned for radioactivity with an automatic and recording instrument (G. Popják, unpublished work).

Chromatography on ion-exchange resins Dowex-2 (formate form) (Dow Chemical Co., Midland, Mich., U.S.A.) and Amberlite IRA-400 (Cl^- form) (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) were made according to the gradient-elution technique of Hurlbert, Schmitz, Brumm & Potter (1954). Details of the elution schedules are shown on Figs. 2 and 3.

Determinations of phosphorus. These were made according to the method of Allen (1940), except that the digestion of the samples was made by combustion with $Mg(NO_3)_2$, and the method was adapted as a microprocedure. To a dried sample of the compound to be analysed in a hard-glass tube 2 drops of a $Mg(NO_3)_2$ solution (10%, w/v, in 95% ethanol) were added and the solvent was evaporated off. The mixture was then ignited by heating in a microflame until all the brown fumes were driven off and a white ash remained; the pyrophosphate which may have been formed was cleft by heating the ash in a stoppered tube with 0·2 ml. of N-HCl in a boiling-water bath for 15 min. After the contents of the tube had cooled down, there were added 0·6 ml. of water, 0·08 ml. of 60% (w/v) perchloric acid, 0·08 ml. of Allen's (1940) amidol (2:4-diaminophenol hydrochloride) reagent and 0·04 ml. of 8·3% (w/v) ammonium molybdate solution. The colour was measured in a 1 ml. microcuvette of 1 cm. light path at 660 mμ in a spectrophotometer 15 min. after addition of the reagents. By this method amounts of orthophosphate as small as 0·02–0·08 μmole could be accurately determined.

RESULTS

Isolation of phosphorylated derivatives of mevalonic acid

The enzymic formation of two new derivatives of mevalonic acid was first observed in short-term (5–10 min.) incubations of the complete enzyme system synthesizing cholesterol from mevalonic acid (cf. Methods and Materials). In such experiments the incubation mixtures were deproteinized with 5% (w/v, final concentration) trichloroacetic acid and the pH of the protein-free filtrate was adjusted to about 8·3 by the addition of $Ba(OH)_2$; the water-insoluble precipitate of barium salts contained, among other things, the new derivatives of mevalonate, which were recognized on paper chromatograms. Further experiments showed, however, that the same derivatives of mevalonic acid were also formed, and in better yield, with the dialysed soluble enzymes alone (without microsomes) in the presence of ATP and Mg^{2+} ions. Subsequently the formation of these substances was studied in incubations containing, in a volume of 3 ml., dialysed soluble enzymes (30–50 mg. of protein), ATP (10 μmoles), potassium phosphate buffer (0·1 M, pH 7·5), $MgCl_2$ (4 mM), nicotinamide (30 mM) and either [1-^{14}C]- or [2-^{14}C]-mevalonate (0·5–2·0 μmoles) as the potassium salt. After incubation of such mixtures for 5–10 min. at 37° the proteins were precipitated by the addition of 3 ml. of ice-cold 10% (w/v) perchloric acid and were removed by centrifuging. The protein-free extracts were neutralized with KOH and the insoluble $KClO_4$ was centrifuged off at 0°. The perchlorate-free extracts were dried from the frozen state *in vacuo* and the dry residues dissolved in 0·5 ml. of water. Chromatography of samples of this solution on Whatman no. 1 paper with the *iso*butyric acid–aq. NH_3 soln.–water system revealed the presence of four radioactive substances whether [1-^{14}C]mevalonate or [2-^{14}C]mevalonate was used as substrate. A typical chromatogram is shown in Fig. 1. The two least

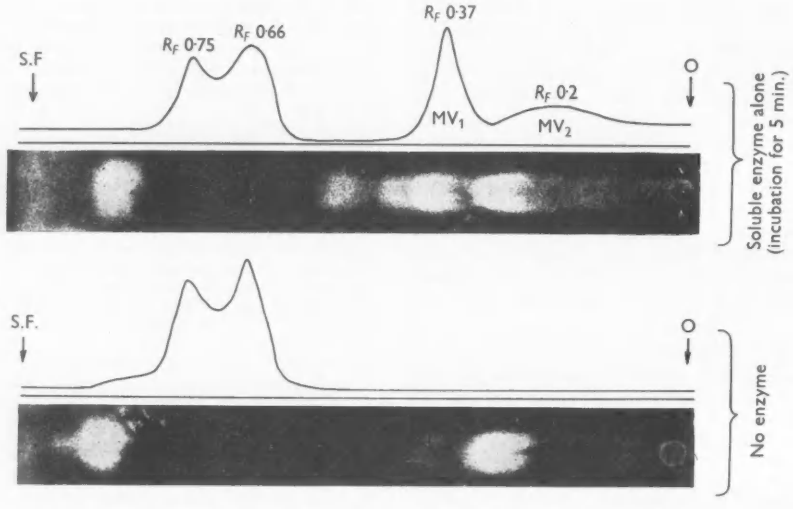

Fig. 1. Paper-chromatographic identification of two derivatives (MV_1 and MV_2) of [^{14}C]mevalonic acid. Photographic prints were made with ultraviolet light and the records of scanning for radioactivity are shown. The lower chromatogram is the control from an incubation mixture containing no enzyme, the upper one shows the appearance of two new radioactive substances after incubation of [2-^{14}C]mevalonate with soluble enzymes, ATP and Mg^{2+} ions for 5 min. at 37°. ○, Origin; S.F., solvent front.

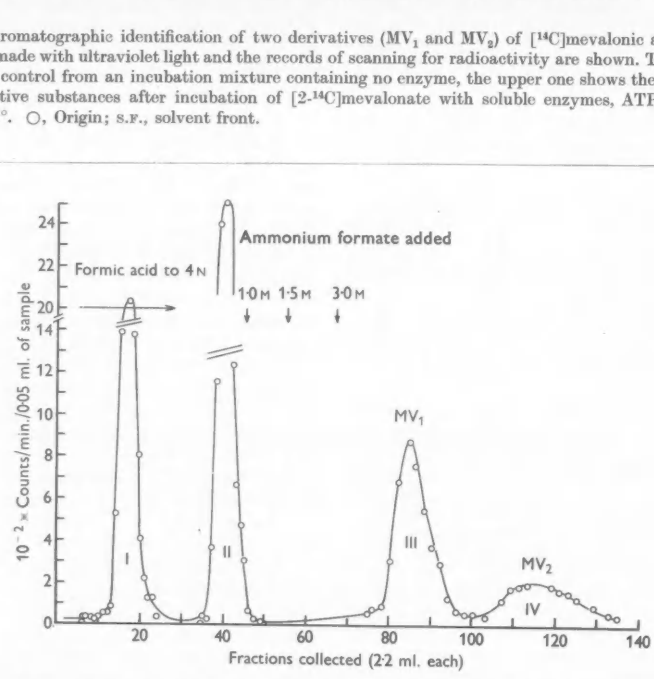

Fig. 2. Results of chromatography on Dowex-2 formate of perchloric acid extract of liver enzymes incubated with [2-^{14}C]mevalonate, according to the gradient-elution technique of Hurlbert, Schmitz, Brumm & Potter (1954). The mixing flask for the eluent contained at the start 100 ml. of water and the reservoir 100 ml. of 4 N-formic acid. At each arrow 50 ml. of ammonium formate solution of the strength indicated, and contained in 4 N-formic acid, was added to the reservoir. Peaks I and II are mevalonic lactone and mevalonic acid respectively, peak III is MV_1 and peak IV is MV_2.

polar substances (R_F 0·75 and 0·66) were identified as mevalonic lactone and mevalonic acid respectively; these two alone were found when mevalonic acid was incubated in the buffer without enzymes. In the *iso*butyric acid–aq. NH_3 soln.–water system there was always found an equilibrium mixture of the lactone and the acid irrespective of whether mevalonic lactone or acid has been applied to the papers. The two more polar substances, MV_1 and MV_2 (R_F 0·37–0·40 and 0·20–0·25), formed from either [1-^{14}C]- or [2-^{14}C]-mevalonic acid, could be detected in significant amounts only when the incubation time was 10 min. or less; at 5–7 min. as much as 30% of the added DL-mevalonic acid could be accounted for in these two new products. The amount of MV_2 was always less than that of MV_1 (cf. Figs. 1 and 2). In incubations lasting longer than 10 min. the amounts of MV_1 and MV_2 gradually diminished, so that after 20 or 30 min. only traces of them could be found, suggesting their utilization for the synthesis of products other than squalene and sterol, which are not formed with the soluble enzymes alone.

The prints of the chromatograms made with ultraviolet light showed that nucleotides were associated with both MV_1 and MV_2 (cf. Fig. 1), but, as subsequent work showed, this was a fortuitous circumstance, adenosine diphosphate (ADP) having an R_F identical with that of MV_1 and ATP an R_F identical with that of MV_2 in the *iso*butyric acid–aq.NH_3 soln.–water system. Furthermore, ADP and ATP were found to migrate in at least three other chromatographic systems to the same places as the two derivatives of mevalonic acid. For this reason we believed at first that nucleotide complexes of mevalonic acid were formed.

The four compounds mevalonic acid and lactone, MV_1 and MV_2, were also separated on Dowex-2 formate ion-exchange resin by gradient-elution technique (Hurlbert *et al.* 1954). Mevalonic lactone was eluted from the Dowex column with the solvent front (water) (Fig. 2, peak I) and mevalonic acid with low concentrations of formic acid (Fig. 2, peak II). Elution with increasing concentrations of ammonium formate (in 4N-formic acid) yielded peaks III and IV, which were identified as MV_1 and MV_2 by paper chromatography. Even after chromatography on this resin ADP and ATP were still associated with MV_1 and MV_2.

Purification of compounds MV_1 and MV_2

For purposes of identification and for testing in enzymic reactions, compounds MV_1 and MV_2, formed from [2-^{14}C]mevalonic acid, were isolated from a large-scale incubation (equivalent to 40 incubations of 3 ml.) by chromatography of the deproteinized mixture on Dowex-2 resin. The compounds MV_1 and MV_2, as obtained from the Dowex column, were finally freed from ADP and ATP as follows. The eluates from the resin were dried from the frozen state; most of the formic acid and ammonium formate was sublimed off in high vacuum, the samples being warmed with an infrared lamp during sublimation. The dried specimen of peak III, containing MV_1, was dissolved in a few millilitres of water and applied to a small charcoal (Nuchar C190, N; West Virginia Pulp and Paper Co., Tyrone, Penn., U.S.A.) column (5 cm. height, 1 cm. in diameter); the charcoal was then washed with water to remove formic acid and ammonium formate. MV_1 was eluted with 7 ml. of 55% pyridine in water; 67% of the applied radioactive substance was thus recovered. After removal of pyridine from the eluate by $CHCl_3$ and ether extractions, MV_1 was chromatographed again on an Amberlite IRA-400 ion-exchange resin (Cl^- form; column height 7 cm., diameter 1·5 cm.). Gradient elution of this column was carried out with 0·015N-HCl containing increasing concentrations of KCl (0·02–0·5M). MV_1 was eluted when the concentrations of KCl in the eluate was between 0·1 and 0·2M (Fig. 3). The dilute solution of the compound, still containing traces of ADP, was dried from the frozen state *in vacuo* and the dry powder extracted with 90% ethanol, in which MV_1 dissolved completely whereas the ADP did not. The solutions of MV_1 now did not absorb light at 260 mμ and were free from inorganic phosphate.

The purified MV_1 was chromatographed again on paper and its R_F values were found to be identical with that of the substance identified as MV_1 in the original perchloric acid extracts of enzyme incubations. On chromatography of the pure MV_1 together with added ADP in the *iso*butyric acid–aq. NH_3 soln.–water system the two substances ran together as originally observed. Since radioactive MV_1 was formed from either [1-^{14}C]- or [2-^{14}C]-mevalonate, the substance contains in all probability all six carbon atoms of mevalonate. The polar behaviour of the substance during chromatography was accounted for by finding that it contained phosphorus. Assuming that MV_1 was a C_6 compound and that no dilution of the [^{14}C]mevalonate with unlabelled material in the incubations occurred, the analyses showed that MV_1 contained one atom of phosphorus per mevalonic acid unit (the actual figure gave 0·8 atom of phosphorus/molecule of mevalonate). The phosphorus is probably present in the compound as a phosphate ester as it proved to be stable to hydrolysis for 7 min. in N-HCl or N-KOH at 100°. As it failed to give a lactone, the esterification probably involves the 5-hydroxy group of mevalonic acid. Judging from these characteristics, MV_1 appears to be identical with the substance isolated by Tchen (1957) and independently by Lynen (1959) from incubations of

yeast enzymes with [^{14}C]mevalonate and claimed to be 5-phosphomevalonate. Lynen (1959) phosphorylated mevalonic acid with $POCl_3$ in pyridine and showed that the product of the reaction was identical with the substance formed in enzyme incubations.

The compound MV_2, obtained as Dowex peak IV (cf. Fig. 2), was separated from contaminating ATP in a manner similar to that used for MV_1. After adsorption to and elution from charcoal with 55% pyridine, the radioactive MV_2 was further chromatographed on Amberlite IRA-400 resin (Cl^- form) as described for MV_1 (Fig. 3). MV_2 was eluted from the resin when the concentration of KCl in the eluate was about 0·2 M. The eluate was dried from the frozen state *in vacuo* and the dry residue extracted with 90% methanol, yielding MV_2 uncontaminated by nucleotides or by inorganic phosphate and which, as judged by its behaviour on paper chromatograms, was radiochemically homogeneous. It had the same properties as the substance identified in the original perchloric acid extracts of enzyme incubations. Phosphorus analysis showed that MV_2 contained two atoms of phosphorus (the actual figure gave 1·82 atoms) per mevalonic acid unit, the calculations being based on the same assumptions as those for MV_1. We cannot offer any further evidence at present on the chemical properties of MV_2; it might be 3:5-diphosphomevalonate, but it is equally possible that the second phosphate group is present in some form other than the tertiary phosphate. The chromatographic behaviour of this substance suggests that it is identical with compound II of Chaykin, Law, Phillips, Tchen & Bloch (1958), who have found this in incubations of yeast extracts with mevalonic acid, ATP and Mg^{2+} ions and suggest that it is mevalonic acid 5-pyrophosphate. For convenience this substance might be referred to as diphosphomevalonate.

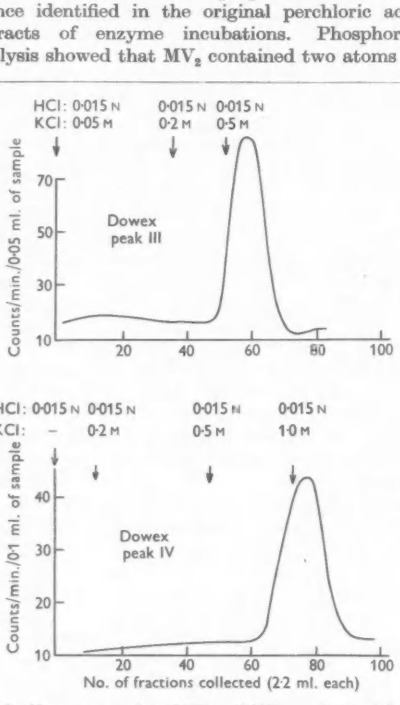

Fig. 3. Chromatography of MV_1 and MV_2, as obtained from Dowex-2 column, on Amberlite IRA-400 (Cl^-) by gradient elution. The mixing flask for the eluent contained at the start 100 ml. of water; at each arrow 50–100 ml. of 0·015 N-HCl containing KCl in the concentrations indicated was added to the reservoir.

5-Phosphomevalonate and diphosphomevalonate as precursors of cholesterol

The two purified compounds were tested as precursors in the biosynthesis of sterol with the complete liver-enzyme system under our standard conditions (Popják *et al.* 1958). As the results in Table 1 show, both 5-phospho-[2-^{14}C]mevalonate and diphospho-[2-^{14}C]mevalonate were more effective precursors of cholesterol than DL-mevalonic acid itself, but both still required the addition of ATP to the incubation mixtures: in the absence of ATP only negligibly small labelling of sterol occurred. These experiments were repeated with three preparations of each compound with identical results.

DISCUSSION

The finding of the same phosphorylated derivatives of mevalonic acid in a mammalian enzyme system

Table 1. *Utilization of 5-phospho-*[2-^{14}C]*mevalonate and of diphospho-*[2-^{14}C]*mevalonate as compared with that of* DL-[2-^{14}C]*mevalonate for cholesterol synthesis*

Each flask contained: soluble dialysed enzymes (1 ml.; 50 mg. of protein); microsome suspension (0·2 ml.); ATP, 30 μmoles; DPNH, 1 μmole; TPN, 1 μmole; ascorbic acid, 30 μmoles; nicotinamide, 120 μmoles; potassium phosphate buffer, pH 7·4, 400 μmoles; Mg^{2+} ions, 16 μmoles; final volume: 4 ml. Incubation was for 1 hr. at 37° with air as gas phase. The substrates were added in equal amounts (0·26 μmole, containing a total of 7600 counts of ^{14}C/min.).

Substrate added	Specific activity of digitonides (counts/min./2 cm.^{2} at infinite thickness)
Mevalonate	1012
5-Phosphomevalonate	1705
5-Phosphomevalonate	30*
Diphosphomevalonate	1552
Diphosphomevalonate	1571
Diphosphomevalonate	40*

* No ATP was added to these incubations.

as were found in yeast extracts (Tchen, 1957; Bloch, 1959; Lynen, 1959) adds weight to the view that the biological synthesis of polyisoprenoid substances proceeds according to similar reactions both in plants and animals and that the key substance is mevalonic acid. The enzyme catalysing the formation of 5-phosphomevalonate from mevalonic acid and ATP, named mevalonic kinase, has been purified by Tchen (1958) from yeast extracts and by Levy & Popják (1959) from extracts of pig liver; apart from some minor differences the enzymes from these two sources have very similar characteristics.

The fact that ATP is still needed for the utilization of either 5-phosphomevalonate or diphosphomevalonate for cholesterol synthesis suggests a further phosphorylating step in the enzymic reactions of mevalonic acid. If the suggestion of Chaykin *et al.* (1958) that diphosphomevalonate is identical with mevalonic acid 5-pyrophosphate is proved correct, then this additional phosphorylation might involve the 3-hydroxy group of mevalonic acid. It seems that at least 3 moles of ATP are needed per mole of mevalonic acid converted into squalene or sterol. Considering that in the synthesis of mevalonic acid from acetate three high-energy bonds of acetyl-coenzyme A are needed (Rudney, 1959) and 6 moles of mevalonate are used for each mole of cholesterol, it follows that synthetic energy equivalent to at least 36 high-energy bonds is needed in the synthesis of one molecule of cholesterol from acetate.

The amounts of the two phosphorylated derivatives of mevalonate that were obtained with the rat-liver enzymes were very variable and critically depended on the length of the incubations, 5–7 min. being the optimum period. After an incubation period of 10 min. or longer the yields became increasingly poor owing to the conversion of mevalonic phosphates into other products. The [^{14}C]mevalonic acid and the [^{14}C]mevalonic acid lactone, isolated by chromatography on Dowex-2 resin after incubating DL-[2-^{14}C]mevalonate with the enzyme mixture for 20 min., were ineffective as precursors of cholesterol. Presumably they represent the unnatural enantiomorphs of the acid and lactone, which, in agreement with the suggestion of Tavormina *et al.* (1956), are not used in sterol biosynthesis.

Since the work described here has been completed, a method based on the adsorption to and elution from charcoal followed by chromatography on Dowex-2 resin has been successfully employed for the large-scale preparation of 5-phosphomevalonate with purified liver mevalonic kinase (Levy & Popják, 1959).

SUMMARY

1. The formation of two phosphorylated derivatives of mevalonic acid by liver enzymes in the presence of adenosine triphosphate and Mg^{2+} ions is described.

2. The two substances were purified by various chromatographic procedures. One of the compounds, MV_1, is thought to be 5-phosphomevalonate. The second, MV_2, contains two atoms of phosphorus per molecule of mevalonate.

3. Both of these substances are converted into cholesterol more efficiently than is DL-mevalonate, but adenosine triphosphate is still needed in this conversion.

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Vitamin A Deficiency and the Ubiquinone and Substance SC Contents of Rat Liver: The Time Factor

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Lowe, Morton & Harrison (1953) found that vitamin A deficiency in rats results in marked changes in the adrenal glands and in the accumulation in the liver of two substances having characteristic ultraviolet-absorption spectra. Heaton, Lowe & Morton (1957) referred to these substances as SA and SC, and the former is now known as ubiquinone (for references see Morton, 1958). Heaton *et al.* (1957) made a detailed comparison of tissues from rats exhibiting avitaminosis A with those of control animals. Ubiquinone was found to occur in many organs but substance SC was detected in normal animals only in kidney, liver and intestine and then in very small amounts. A sharp rise occurred, however, in the amounts of both ubiquinone and substance SC in the livers of rats suffering from frank vitamin A deficiency. Green, Lowe & Morton (1957) subjected rats to the double hazard of vitamin A deficiency and a high intake of dietary cholesterol. The latter neither diminished nor enhanced the rises in liver ubiquinone and substance SC observed in uncomplicated vitamin A deficiency. A hypothesis that the accumulation of these two new substances was a necessary part of the deficiency syndrome was tested on fowl and on guinea pigs. Lowe, Morton, Cunningham & Vernon (1957) found in fowl that the ubiquinone and substance SC of liver did not rise in vitamin A deficiency and Cunningham, Morton & Phillips (unpublished work) observed that the ubiquinone content of guinea-pig liver was also unresponsive to vitamin A deficiency. Such notable differences between species make it necessary to investigate further the effects shown in the rat. Thus it seems desirable to determine at what stage in the development of avitaminosis A the changes occur in storage or metabolism of ubiquinone and substance SC. In the earlier work (Heaton *et al.* 1957) the rats deprived of vitamin A were not killed until there was manifest xerophthalmia and a severe decline in body weight.

In the present work food consumption by individual rats was measured throughout the period of experiment. There is general agreement that vitamin A deprivation reduces appetite but quantitative information is lacking on the rate at which food consumption falls off as the deficiency syndrome develops. Sampson, Dennison & Korenchevsky (1932) found that during the period when the growth of deprived rats slows to a halt the decrease in appetite is only moderate. Anorexia can be no more than a partial explanation of the diminishing growth. Indeed it seemed that (in relation to body weight) the deprived rats ate rather more than rats on a complete diet. This continued until a late stage when the frankly deficient rats exhibited a marked loss of appetite. Patterson, McHenry & Crandall (1942) observed that with vitamin A-deprived animals on a weight plateau for 3 weeks the food consumption declined linearly with time from 16 to 7 g. of diet/rat/day.

EXPERIMENTAL

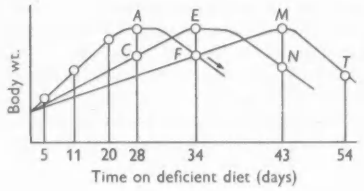
Examination of tissues. Livers were saponified as described in previous papers (Mervyn & Morton, 1959) and the unsaponifiable extracts were chromatographed on alumina partially deactivated to Brockman grade 3. Elution by means of light petroleum containing increasing proportions of diethyl ether yielded in succession fractions rich in hydrocarbons, in ubiquinone, in sterol and the substance SC.

Animals. Albino rats were purchased from A. Tuck and Sons, Rayleigh, Essex and at about 30 days of age they were placed on a diet with the following percentage composition: sugar, 65; casein (ethanol-extracted), 18; arachis oil, 5; dried brewer's yeast, 8; salt mixture (Hubbell, Mendel & Wakeman, 1937), 4. Food and water were given *ad lib.*

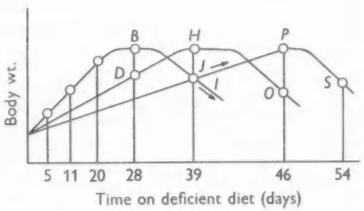
Experimental plan. The rats (59 males, 59 females) were given the deficient diet for varying lengths of time. Two groups of 15 animals were killed 5 days after weaning, two groups of 10 were killed 11 days after weaning and two groups of 10 were killed 20 days after weaning. These groups are designated as the 5-day, 11-day and 20-day groups respectively. The remaining 48 animals (24 males and 24 females) were housed in individual cages from weaning. Daily food consumption for the 48 animals was measured by difference. A weighed quantity of the diet was measured out each morning and the amount left over after 24 hr. was weighed after recovery of spilled food from filter paper placed about 1 in. below the wire-mesh floor of the cage.

In an experiment of this kind, the time at which individual animals reach a given stage of deficiency varies rather widely. All the animals were weighed frequently and those which reached a given stage were taken together. Figs. 1*a*

* Seconded from Chemistry Division, Department of Agriculture, Science Service, Ottawa, Canada.



(a)



(b)

Fig. 1. Each open circle represents a group of rats killed at a definite stage in the growth curve. Circles without letters refer to groups of rats for which individual food consumptions were not measured. Each lettered circle refers to three rats for which individual food intakes were measured daily. (Diagrammatic only.) (*a*) Male rats; (*b*) female rats.

and (*b*) illustrate the manner in which animals were selected for examination. Each letter in the diagrams represents a group of three animals killed and studied as a group. Thus point *C* represents three male rats killed at 28 days; *D* represents three female rats killed at 28 days; *J* represents three female rats killed after 39 days, all three groups without slackened growth. Groups *C*, *D* and *J* can be collectively taken as representing the period a little before the weight plateau. Similarly, *A*, *B*, *E*, *H*, *M* and *P* each represents three rats at the weight plateau. *F*, *I*, *N*, *O*, *T* and *S* each represents three rats declining in weight and approaching the terminal phase of deficiency. Figs. 2 (*a*) to 2 (*d*) refer to means for three rats and show how weight curves correspond with food-intake curves.

RESULTS

Weight increases in relation to food consumption. It is evident from Figs. 2 (*a*) to 2 (*d*) that although the rats were all from one colony and were given the same diet, the growth rate varied considerably. A few rats of each sex reached a growth (i.e. weight) plateau after 21 days on the deficient diet (groups *A* and *B*) but others required about 37 days (groups *M* and *P*). This difference might be the result of differences in liver stores of vitamin A at weaning or possibly differences in metabolic losses of vitamin A.

Occasionally an animal would scatter food so that it was doubtful whether the apparent intake corresponded closely with the amount actually eaten; all such cases were left out of the calculations but very few of the 1800 daily food consumptions measured had to be rejected.

The daily intake of food increased steadily to a maximum which in most cases occurred 11–13 days after weaning. In those animals which reached a plateau in body weight earlier than about 27 days after weaning, food consumption began to fall after about the fourteenth day. Animals which took longer to reach stationary body weight did not begin to lose appetite until about 4 days before the appearance of the weight plateau. From then on they steadily ate less.

Six groups of three animals were maintained on the vitamin A-deficient diet until an unmistakable loss in body weight had occurred. A marked decline in food consumption occurred at or near the end of the plateau period. Appetite waned before growth ceased but an animal was not badly 'off its food' during the variable and some times lengthy period before the terminal stages of the deficiency syndrome. There was a variable lag between loss of appetite and loss of weight. Patterson *et al.* (1942) observed that vitamin A-deficient rats at the plateau stage of the deficiency syndrome contained 6 % more body water than pair-fed control rats.

Agnew & Meyer (1956) have thrown light on the loss of appetite shown by vitamin A-deficient rats. In the hypothalamus of normal rats there appears to be an area which acts as a 'satiety' brake, which, in response to metabolic stimuli, inhibits an otherwise constantly acting feed mechanism. By subjecting vitamin A-deficient rats, which had stopped growing, to a hypothalamic injury which destroyed the satiety centre, a weight increase of 70–200 g. occurred. The appearance in many of the rats in the present experiment of loss of appetite and slackened growth after 11–13 days on the vitamin A-deficient diet suggests that an application of the satiety brake may be an early aspect of the deficiency syndrome.

Effects on the liver. Table 1 shows how body weight and liver weight changed during the course of the experiment. In the youngest animals the liver accounted for a larger proportion of the total weight than in the somewhat older animals, but beyond 3 weeks after weaning the proportion became fairly constant and showed no great difference before, during or after the weight plateau. An animal which lost weight suffered a roughly proportionate decline in liver weight.

Table 2 shows how the amounts of hydrocarbon, of ubiquinone, substance SC and sterol in liver tissue varied during the course of the experiment.

DISCUSSION

The original work (Lowe *et al.* 1953; Heaton *et al.* 1957) on the effects of vitamin A deficiency on ubiquinone (SA) and substance SC levels in rat tissues was carried out on frankly avitaminotic animals. The ubiquinone content of the livers rose from 120 μg./g. in the control rats to 283 μg./g. in the deficient animals. For stock-colony animals the level was 78 μg./g. and substance SC was just

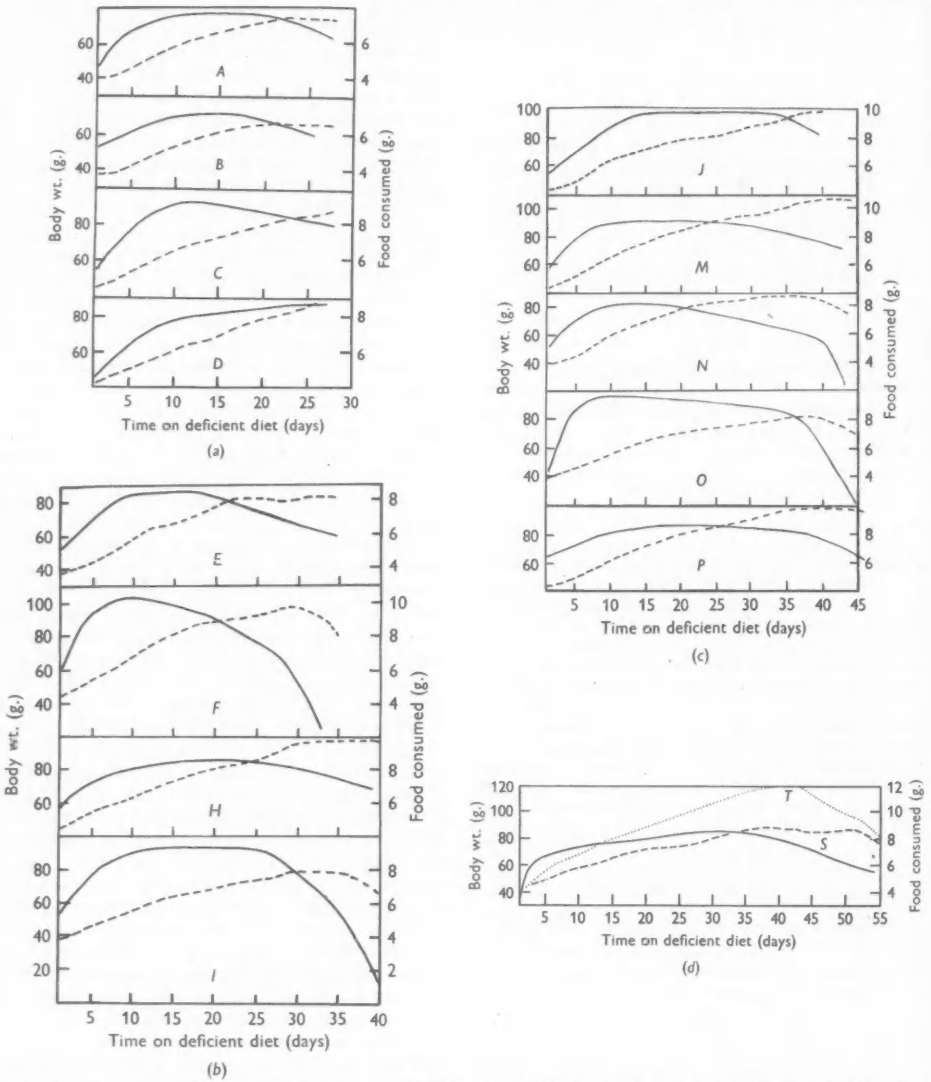


Fig. 2. In each diagram the full curve is food consumption (g./day) plotted against days on the vitamin A-deficient diet and the broken curve shows body weight (g.) plotted against days. Each group consisted of three animals. These diagrams should be read in conjunction with Fig. 1 and Table 1. The dotted line in (*d*) refers to body weight in group *T* for which the food consumption was not recorded fully.

detectable. The level of substance SC in the control animals (with small vitamin A reserves) was 7·19 arbitrary units/g. and in the avitaminotic animals 15·5 units/g. (15·5 units/g. = 170 μg./g. on basis of $E^{1\%}_{1\,cm.}$ 275 mμ = 90).

In the present work (Table 2) the liver ubiquinone level began at 82 μg./g. and rose steadily to a value of 300 μg./g. at the weight plateau, and still further to 410 μg./g. of liver when the rats were rapidly losing weight. Heaton *et al.* (1957) found that the sterol content of liver rose from 2·03 mg./g. of liver in the stock-colony rats to 2·19 mg./g. in the control rats and 2·78 mg./g. in the avitaminotic rats. In the present series of determinations the rats killed

Table 1. *Effects of vitamin A deficiency in relation to the stage reached in the syndrome*

Group	Sex	Average body wt. (g.)	Liver wt. (as % of body wt.)	Unsap. matter (as % of liver wt.)	Ubiquinone (μg./g. of liver)
			Growing		
5-day	M.	46·8	8·15	0·298	85·9
	F.	48·2	7·43	0·306	78·8
	Mean	**47·5**	**7·79**	**0·302**	**82·3**
11-day	M.	68·8	6·18	0·374	132
	F.	63·5	6·25	0·374	140
	Mean	**66·2**	**6·21**	**0·374**	**136**
20-day	M.	84·9	5·47	0·404	227
	F.	81·7	5·78	0·402	187
	Mean	**83·3**	**5·62**	**0·403**	**207**
C	M.	88·7	4·67	0·410	220
D	F.	87·3	4·58	0·430	117
J	F.	103	4·96	0·456	398
	Mean	**93·0**	**4·74**	**0·432**	**245**
			Weight plateau		
A	M.	74·3	4·89	0·455	334
E	M.	82·0	4·89	0·392	245
M	M.	105·0	4·36	0·411	253
B	F.	63·7	4·85	0·553	326
H	F.	84·7	5·28	0·448	313
P	F.	97·0	4·56	0·475	325
	Mean	**84·5**	**4·80**	**0·456**	**299**
			Losing weight		
F	M.	82·0	4·58	0·381	297
N	M.	72·0	4·53	0·615	416
T	M.	81·0	5·42	0·450	413
I	F.	83·5	3·91	0·683	660
O	F.	68·0	3·97	0·574	315
S	F.	71·7	5·06	0·420	355
	Mean	**73·0**	**4·6**	**0·521**	**409**

Table 2. *Minor constituents of rat liver observed as vitamin A deficiency develops*

Stage ...	5-day	11-day	20-day	28-day	Weight plateau	Post-plateau
Hydrocarbon fraction (μg./g.)	72	62	60	116	184	121
Absorption at 260 mμ (units/g.)*	0·78	0·89	1·43	1·54	1·79	2·67
Ubiquinone (μg./g.)	82·3	136	207	245	299	409
Ubiquinone (mg./100 g. body wt.)	0·64	0·85	1·16	1·18	1·44	1·88
Substance SC (μg./g.)†	7·33	6·55	9·32	32·3	32	45·2
Substance SC (mg./100 g. body wt.)	0·06	0·041	0·052	0·155	0·154	0·21
Sterol (mg./g.)	1·85	2·27	2·26	2·20	2·43	2·81
Sterol (mg./100 g. body wt.)	14·6	14·05	12·6	10·4	11·7	12·7

* The units refer to $E^{1\%}_{1\,cm.}$ 260 mμ for the fraction, multiplied by its weight in mg.
† The basis of this calculation is that $E^{1\%}_{1\,cm.}$ 275 mμ = 90 for the purest substance SC yet obtained.

after 5 days on the vitamin A-deficient diet showed 1·85 mg./g. of liver and 2·2–2·3 mg./g. for the 11- to 28-day periods, and 2·34 mg./g. at the plateau and 2·81 mg./g. of liver when weight was rapidly decreasing. This reproduces the earlier picture.

The content of substance SC in liver rose substantially as the vitamin A deficiency progressed, whether the result was expressed as μg./g. of liver or mg./100 g. body weight. It did not, however, reach in the present experiment the very high value found by Heaton *et al.* (1957). This result may be accounted for if the sharp rise in substance SC is characteristic of the terminal stages of vitamin A deficiency. In the present study the 'post-plateau' animals were not all as severely avitaminotic as those of Heaton *et al.* Table 2 indicates that the liver-hydrocarbon content also rose in vitamin A deficiency. Two criteria for hydrocarbons may be used in this context; one is the weight of unsaponifiable matter eluted by means of light petroleum, and the other is the intensity of absorption at 260 mμ. The former criterion reflects the sum of saturated and unsaturated hydrocarbons and the latter may measure one or more of the unsaturated hydrocarbons. A steady increase in absorption at 260 mμ occurred as the vitamin A deficiency progressed and a roughly linear relationship was noted between this increase and the rise in ubiquinone content. A rise of 0·1 unit/g. at 260 mμ was accompanied by an average rise of nearly 20 μg./g. in liver ubiquinone. Since ubiquinone has a $-C_{50}H_{81}$ unsaturated side chain, the parallel increase in a free unsaturated hydrocarbon fraction is interesting and possibly significant.

It was in some ways surprising that the phenomena of increases in ubiquinone and substance SC in the livers of rats suffering from avitaminosis A were not reproduced in fowls (Lowe *et al.* 1957) nor in guinea pigs (Cunningham, Morton & Phillips, 1959, unpublished work). The present work shows that whatever the explanation of the species differences, the observations on the rat are consistent with progressive rises in liver ubiquinone and substance SC concentration with increasing time on the deficient diet.

SUMMARY

1. Weanling rats placed on a vitamin A-deficient diet reached a weight plateau after a variable time. Food consumption began to fall after about 14 days in those animals which reached the plateau earlier than 27 days after weaning. Animals which took longer to become stationary in weight began to lose appetite about 4 days before growth ceased. Anorexia became marked only in animals rapidly losing weight.

2. Groups of deprived animals were killed at 5, 11, 20, 28 and 39 days while still growing; other groups were killed at the weight plateau and others at a time when they were losing weight. Chromatographic examinations of liver unsaponifiable matter showed: (*a*) a rise in sterol content only at or beyond the weight plateau; (*b*) a rise in substance SC which became marked when growth had slackened or ceased; (*c*) a progressive rise in ubiquinone; the increase was already definite in the 11-day group and when the phase of declining weight was reached the concentration averaged five times the concentration seen in the group of rats killed 5 days after weaning. The amount of a hydrocarbon fraction showing selective absorption at 260 mμ also increased steadily with the rising ubiquinone concentration.

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Unsaponifiable Constituents of Rat Tissues in Relation to Vitamin K Status

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This paper is concerned with the effects of vitamin K deficiency on minor constituents of the unsaponifiable matter from selected rat tissues.

A first approach has been to produce a conditioned vitamin K_2 deficiency by adding to the diet sufficient sulphadiazine to exert a strong bacteriostatic effect on the flora of the gut. This experiment was performed twice, and in each case the sexes were studied separately. When the sulphadiazine-fed animals had become hypoprothrombinaemic they were killed and the liver, kidney and intestinal, tissues were removed. These and control tissues were saponified and the unsaponifiable fraction was studied.

A second approach was to place all the animals on a basal diet free from vitamins K_1 and K_2. The control animals were given this diet plus a small amount of menadione (2-methyl-1:4-naphthaquinone, vitamin K_3) and the deprived animals were given the basal diet plus 1% of sulphadiazine to inhibit bacterial synthesis of vitamin K_2. After 21 days all the animals were killed and various tissues were examined.

A third approach was to incorporate in a normal diet for 26 days the vitamin K antagonist dicoumarol and to compare animals so treated with control animals on the unsupplemented stock diet.

A fourth approach was to maintain rats for 45 days on a vitamin A-deficient diet until the reserves had been heavily depleted. Some animals were then killed and the tissues examined. The remaining rats were divided into two groups, one of which was given vitamin A and the other sulphadiazine. In this way it was hoped to show any effects due to the double stress of vitamin A deficiency and vitamin K deficiency. Particular attention was given to ubiquinone concentrations.

METHODS

Animals. Weanling hooded rats reared in the Department were used.

Animal diets. (*a*) The stock diet of cubes (diet no. 4, British Extracting Co., Bromborough, Cheshire) was ground to a powder and fed as such. (*b*) Sulphadiazine (1%) or dicoumarol (L. Light and Co. Ltd.; 0·003%) was added to the powdered diet and thoroughly mixed. (*c*) A basal diet free from vitamin K_1 had the following percentage composition: sucrose 73; casein (vitamin-free), 18; arachis oil 3; cod-liver oil (1000 i.u. of vitamin A/g.) 2; salt mixture (Hubbell, Mendel & Wakeman, 1937) 4. To this diet was added a vitamin mixture (2 g./kg. of diet) consisting of (g.): calcium pantothenate 0·835, *p*-aminobenzoic acid 0·167, thiamine hydrochloride 0·0835, biotin 0·0015, choline chloride 32·326, folic acid 0·0167, inositol 16·163, nicotinic acid 0·167, pyridoxine 0·067, riboflavin 0·167. (*d*) A basal diet free from vitamin A contained (%): sucrose 65; casein (vitamin-free) 18; Marmite 8; arachis oil 5; salt mixture (Hubbell *et al.* 1937) 4. Vitamin mixture was added as above (2 g./kg. of diet).

Determination of prothrombin time. The effectiveness of the sulphadiazine supplement in producing vitamin K_2 deficiency was determined by measuring the prothrombin time, following the method of Quick (1937, 1940). Plasma from the blood of stock colony rats was diluted with aq. 0·85% sodium chloride solution to give concentrations of 10, 20, 30 and 50% (v/v) of plasma. A thromboplastin activity curve was obtained (Bacto thromboplastin, Difco Laboratories). Blood was obtained from four animals in each group (2·7 ml., by heart puncture) and to it was added 0·1 M-sodium oxalate (0·3 ml.). After centrifuging, the plasma was at once drawn off and the clotting time was determined within 6 hr. (see Tárnoky, 1958).

Examination of tissues. The methods of saponification, extraction of unsaponifiable matter and chromatography on acid-washed alumina partially deactivated to Brockman grade 3 have been described in earlier papers (cf. Mervyn & Morton, 1959). The substances under study were: (i) a hydrocarbon fraction very feebly adsorbed on alumina and exhibiting an inflexion near 260 mμ. It is probable that the weight of this fraction is a measure (in the main) of saturated hydrocarbons and that the intensity of absorption at 260 mμ is a measure of unsaturated material; (ii) ubiquinone (for references see Morton, 1958); (iii) substance SC (Lowe, Morton & Harrison, 1953; Heaton, Lowe & Morton, 1957); (iv) vitamin A; (v) cholesterol and 7-dehydrocholesterol; (vi) substance SF or 3-formylindole (Morton & Fahmy, 1958).

RESULTS

Sulphadiazine added to stock diet

From weaning at the age of 21 days, rats in groups *A*, *C*, *E* and *G* received the diet of ground cubes and those in groups *B*, *D*, *F* and *H* the same diet with the addition of 1% of sulphadiazine. It will be seen from Tables 1 and 2 that in both sexes

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the sulphadiazine supplement markedly depressed the final body weight and the weights of liver and kidney. Spleen weights were reduced to as little as 30% of those of the control animals. The prothrombin times indicated very marked decreases in prothrombin concentration as a result of the ingestion of sulphadiazine. It was not necessary to confirm the conditioned deficiency in this way in all the experiments as the changes in tissue weights were unmistakable. Table 3 shows the results of chromatographic separations and spectrophotometric analyses on liver tissue. The ubiquinone content of liver did not respond to sulphadiazine added to the food. There was no additional mobilization of vitamin A and no significant change in sterol content. The amount of substance SC did not

Table 1. *Comparison of rats kept for* 36 *days on a diet containing* 1% *of sulphadiazine, with rats on a normal diet*

	Mean weight (g.)			
Diet	Sulphadiazine added		Normal	
Group	*B* (7 males)	*D* (8 females)	*A* (8 males)	*C* (8 females)
Initial	36	40	36	40
Final	72	84	98	101
Liver	3·17	3·84	4·08	4·18
Liver (% of body wt.)	4·44	4·55	4·17	4·14
Kidney	0·40	0·43	0·46	0·45
Spleen	0·14	0·26	0·57	0·71
Intestine	6·06	7·80	7·60	8·40
Prothrombin (% of control)	22	18·5	100	100

Table 2. *Comparison of rats kept for* 29 *days on a diet containing* 1% *of sulphadiazine with rats on a normal diet*

	Mean weight (g.)			
Diet	Sulphadiazine added		Normal	
Group	*F* (8 males)	*H* (8 females)	*E* (8 males)	*G* (8 females)
Initial	43·6	38·3	39·5	37·3
Final	103	89	157	127
Liver	5·44	4·91	8·58	7·26
Liver (% of body wt.)	5·28	5·52	5·46	5·72
Kidney	0·54	0·47	0·75	0·66
Spleen	0·34	0·27	1·21	0·77
Intestine*	7·0	6·9	9·9	7·7

* Includes in this case stomach and intestine.

Table 3. *Comparison of unsaponifiable constituents from liver of vitamin* K_2*-deficient rats and control rats*

	Males		Females		Males		Females	
Group	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Sulphadiazine	Nil	1%	Nil	1%	Nil	1%	Nil	1%
Mean liver wt. (g.)	4·08	3·17	4·18	3·84	8·58	5·44	7·26	4·91
Unsaponifiable matter (%)	0·41	0·41	0·56	0·47	0·33	0·38	0·31	0·43
Ubiquinone (μg./g. of liver)	110	148	210	175	116	109	113	115
Substance SC (units/g. of liver)*	5·2	7·3	2·7	3·2	Not measured			
Vitamin A (i.u./g. of liver)	78	116	91	107	19	68	55	75
Sterol (mg./g. of liver)	2·4	2·46	3·26	2·41	2·02	2·12	2·05	2·07
Hydrocarbon (mg./g. of liver)	0·03	0·10	0·13	0·16	0·06	0·12	0·07	0·12
'Petrol 260 mμ'† (units/g. of liver)	2·1	2·4	2·2	2·2	0·81	1·07	1·04	1·03
Substance SF (3-formylindole)‡ (units/g. of liver)	3·06	Trace	2·41	5·14	1·37	0·69	0·81	0·66

* 1 mg. = 96 units (1 unit = $E^{1\%}_{1\,cm.}$ at 275 mμ × wt. in mg.).

† Eluted from alumina by light petroleum and showing an inflexion at 260 mμ. The unit is $E^{1\%}_{1\,cm.}$ 260 mμ × wt. of fraction in mg.

‡ 1 unit = $E^{1\%}_{1\,cm.}$ 282 mμ × wt. in mg.

Table 4. *Comparison of unsaponifiable constituents from kidneys of vitamin K_2-deficient rats and control rats*

Substance SC was not detected in any group. 7-Dehydrocholesterol was present in traces.

	Expt. 1		Expt. 2	
No. of animals	16	15	16	16
Sulphadiazine in diet	Nil	1%	Nil	1%
Mean kidney wt. (pair; g.)	0·91	0·83	1·40	1·02
Kidney constituents				
Unsaponifiable (%)	0·72	0·65	0·52	0·52
Hydrocarbon (mg./g.)	0·12	0·15	0·04	0·025
Ubiquinone (μg./g.)	81	81	100	72
Vitamin A (i.u./g.)	8·3	4·2	Trace	Trace
Sterol (mg./g.)	4·7	4·2	3·9	3·6

Table 5. *Comparison of unsaponifiable constituents from intestines of vitamin K_2-deficient rats and control rats*

7-Dehydrocholesterol was detected in each case.

	Males		Females		Males		Females	
Group	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Sulphadiazine	Nil	1%	Nil	1%	Nil	1%	Nil	1%
Mean wt. of intestine (g.)	7·6	6·1	8·4	7·8	9·9	7·0	7·7	6·9
Unsaponifiable matter (%)	0·37	0·36	0·39	0·32	0·31	0·41	0·40	0·4
Ubiquinone (μg./g.)	47	48	39	53	41	41	54	43
Sterol (mg./g.)	2·31	2·57	2·44	2·36	2·45	2·59	2·53	2·62
Hydrocarbon (mg./g.)	0·033	0·053	0·019	0·064	0·051	0·029	0·192	0·109

Table 6. *Comparison of control rats and vitamin K_2-deficient rats*

Overall average values are given.

	Liver		Kidneys		Intestine	
	Control	K_2-deficient	Control	K_2-deficient	Control	K_2-deficient
Mean wt. of tissue (g.)	6·03	4·34	1·16	0·92	8·42	6·94
Unsaponifiable matter (%)	0·403	0·422	0·617	0·580	0·370	0·372
Ubiquinone (μg./g.)	137	137	91	76	45	44
Vitamin A (i.u./g.)	61	92	8·3	4·2	Not detected	
Sterol (mg./g.)	2·43	2·27	4·29	3·90	2·43	2·54
Hydrocarbon (mg./g.)	0·07	0·12	0·09	0·08	0·07	0·06

Table 7. *Comparison of rats on a vitamin K-deficient diet with 1% of sulphadiazine for 21 days with control rats given menadione*

The basal diet was diet (*C*) described under Methods. For groups *A* and *B* menadione (5·3 mg./kg.) was added. Substance SF (3-formylindole) was detected in all cases.

	Vitamin K_3-supplemented		Vitamin K-deficient (sulphadiazine added)	
Group	*A*	*B*	*C*	*D*
Sex	Male	Female	Male	Female
Survivors (no. out of 8)	8	8	7	5
Mean initial wt. (g.)	32	32	31	33
Mean final wt. (g.)	89·8	81·5	46·6	52·2
Mean liver wt. (g.)	4·68	4·22	2·85	3·29
Liver constituents				
Unsaponifiable matter (%)	0·308	0·33	0·405	0·375
Ubiquinone (μg./g.)	107	112	127	118
Vitamin A (i.u./g.)	69·6	64·2	26·0	28·8
Sterol (mg./g.)	2·13	2·20	2·21	2·26
Hydrocarbon (mg./g. of liver)	0·42	0·38	0·21	0·12

change. Indeed the only indication of change is a small apparent increase in the hydrocarbon content of the unsaponifiable matter. Tables 4 and 5 show a similar lack of response to conditioned vitamin K_2 deficiency in the minor constituents of kidney and intestine unsaponifiable matter. Table 6 presents the overall picture for both sexes in the two experiments. It is clear that the depression in tissue weight is not paralleled by any marked changes in unsaponifiable constituents.

Sulphadiazine added to a diet deficient in vitamin K_1

In this experiment the rats in groups *A* and *B* grew normally, but the vitamin K-deficient rats increased in weight only very slowly. Three females and one male died and were found at post-mortem examination to show haemorrhages. The experiments were therefore terminated after 21 days. Table 7 shows in the deficient animals a very marked retardation of growth and small livers. The liver weight expressed as a percentage of body weight rose, however, from 5·2 to 6·3. Examination of the liver unsaponifiable fractions showed that the vitamin A reserves had been substantially reduced in the vitamin K-deficient animals. If the findings are calculated in terms of i.u./liver, the average vitamin A content fell from about 300 to 85 in the vitamin K-deficient animals. There was no such decrease in ubiquinone content and the sterol content expressed as mg./g. of liver was unchanged. The menadione-supplemented animals showed a somewhat higher content of liver hydrocarbon.

Dicoumarol added to a stock diet

Ten female weanling rats were divided into two groups having the same aggregate weight. The control animals were given the stock diet of ground cubes *ad lib.* and the others received this diet supplemented with dicoumarol. The treatment significantly (P 0·01) reduced the rate of growth and the final body weight, but no gross lesions were seen. Table 8 shows that the minor constituents of liver were not very much affected by the treatment. The vitamin A was low even in the control rats, so that the low value for the dicoumarol-treated rats is probably not significant.

Combined vitamin K and vitamin A deficiency

A number of weanling rats were placed on a vitamin A-deficient diet for 45 days. At that stage some were killed and it was confirmed that all the liver reserves of vitamin A had disappeared. Fourteen remaining animals were then divided into two groups. One was given the original diet supplemented with vitamin A (100 i.u./rat/day) and the other group was given the same diet supplemented with sulphadiazine (1 %). After 16 days the surviving animals were killed; five of the first group failed to recover after vitamin A therapy and four of the animals receiving sulphadiazine died. Table 9 summarizes the results obtained on the survivors. The rise in ubiquinone content of rat liver in vitamin A

Table 8. *Comparison of rats kept for 26 days on a diet containing dicoumarol (30 mg./kg.) with control rats*

Five female rats were in each group.

	Control	Dicoumarol-treated
Mean initial wt. (g.)	36	37
Mean final wt. (g.)	117	108
Mean liver wt. (g.)	5·87	6·11
Liver constituents		
Unsaponifiable matter (%)	0·413	0·518
Ubiquinone (μg./g.)	160	176
Vitamin A (i.u./g.)	25	31·4
Sterol (mg./g.)	2·24	2·39
Hydrocarbon (mg./g.)	0·88	1·72

Table 9. *Comparison of vitamin A-deficient rats with rats suffering from combined vitamin A and vitamin K deficiency and rats given a vitamin A supplement*

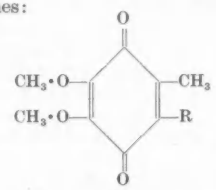
Group	1	2	3
Treatment	Vitamin A-deficient animals killed at 45 days	Vitamin A-supplement given after 45 days	Vitamin A- and K-deficient after 45 days (sulphadiazine supplement given)
Body wt. after 45 days on vitamin A-deficient diet (g.)	81	73	93
Body wt. after 61 days (g.)	—	132	80
Liver wt. (% of body wt.)	4·46	4·54	3·75
Liver constituents			
Unsaponifiable matter (%)	0·655	0·632	0·609
Ubiquinone (μg./g.)	511	190	660
Substance SC (units/g.)*	9·85	3·9	12·0
Substance SC (units/liver)*	35·6	23·4	36
Sterol (mg./g.)	2·93	2·75	2·93
Hydrocarbon (mg./g.)	0·86	0·38	0·89
Vitamin A (i.u./g.)	Nil	62	Nil

* See Table 3 for explanation of units.

deficiency continued when the diet lacked both vitamin A and vitamin K but was reversed when vitamin A was provided and the deficiency syndrome corrected. Similarly, the rise in substance SC was reversed. No marked change in sterol content occurred as a result of the double hazard of vitamin A and vitamin K deprivation.

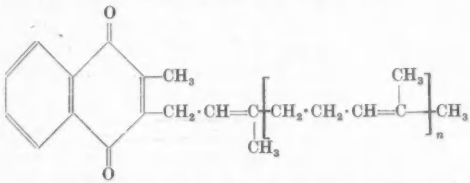
DISCUSSION

The present work is an extension of earlier work on the distribution of ubiquinones (for references see Morton, 1958). These compounds are dimethoxybenzoquinones:



where R is a polyisoprenoid grouping. The ubiquinones of heart and probably of kidney and liver have a $C_{50}H_{81}$ side chain.

Vitamin K_2 (McKee, Binkley, Thayer, MacCorquodale & Doisy, 1939; Binkley, McKee, Thayer & Doisy, 1940), has the structure:



where $n = 5$. Isler, Rüegg, Chopard-dit-Jean, Winterstein & Wiss (1958) have further purified vitamin K_2 and have found its formula to be $C_{46}H_{64}O_2$, so that $n = 6$ and the side chain has 35 carbon atoms. These authors have synthesized vitamin K_2 and a number of lower homologues. Careful determinations of intensities of ultraviolet absorption have shown that the absorption spectrum is uninfluenced by the length of the polyisoprenoid side chain in the sense that the molecular-extinction coefficients are the same through the series (the $E^{1\%}_{1\,cm.}$ values are, of course, higher for the lower members). It is convenient to select the 248 mμ absorption peak to illustrate this; if the side chain is C_{10} (geranyl), $\epsilon_{max.}$ 248 mμ = 19 000 ($E^{1\%}_{1\,cm.}$ 617), and if the side chain is C_{35}, $\epsilon_{max.}$ = 18 900 ($E^{1\%}_{1\,cm.}$ 292).

Francis, Madinaveitia, Macturk & Snow (1949) and Snow (1952) isolated from a strain of *Mycobacterium tuberculosis* a vitamin K_2 homologue as an uncrystallized oil ($E^{1\%}_{1\,cm.}$ 248 mμ 241; C, 85·1; H, 10·27 %). Noll (1958), using *M. tuberculosis* (Brevannes), obtained vitamin K_x (m.p. 58–59°; $E^{1\%}_{1\,cm.}$ 249 mμ 240; C, 85·24; H, 10·27 %), which agreed well with the properties of Snow's compound.

If the molecular-extinction coefficient of vitamin K_x is 18 900, as in vitamin K_2, its molecular weight should be 18 900/24 = 787. Vitamin K_x may be written $C_{11}H_7O_2{\cdot}R$, so that R = 616 and $C_{45}H_{73}$ = 613 ($C_{50}H_{90}O_2$ requires C, 85·65; H, 10·27 %). Noll considers that K_x contains seven to eight isoprenoid units, but the $E^{1\%}_{1\,cm.}$ value points more definitely to a C_{45} side chain with nine double bonds. A hydrocarbon $C_{45}H_{76}$ was isolated from liver by Channon, Devine & Loach (1934) and the formula was confirmed by Dimter (1941, 1942).

The ubiquinones with polyisoprenoid side chains thus resemble the vitamins K of microbiological origin, and the existence of a $C_{45}H_{76}$ hydrocarbon hitherto unrelated to substances of known biochemical interest makes it necessary to explore the possibility of biogenetic or metabolic interrelationships.

The best-known manifestation of vitamin K deficiency is the prolongation of the clotting time of blood, attributed to a reduced concentration of prothrombin (Dam, Schønheyder & Tage-Hansen, 1936), a compound which is made in the liver (Warner, Brinkhous & Smith, 1938; Warren & Rhoads, 1939). According to Quick & Collentine (1950, 1951), vitamin K_1 may be the prosthetic group of an enzyme occurring in liver and concerned in the synthesis of prothrombin, but vitamin K does not itself appear to be an integral part of prothrombin (Dam, Glavind, Lewis & Tage-Hansen, 1938).

Lowe *et al.* (1953) found that vitamin A deficiency brought about in rats a marked increase in two substances, SA and SC, found in small amounts in liver and some other tissues. This phenomenon does not occur in all species. Substance SA has since been called ubiquinone (see Morton, 1958) and coenzyme Q (Lester, Crane & Hatefi, 1958).

The whole problem of lipid cofactors participating in oxidation processes is being studied in many laboratories. Inasmuch as, in the rat at any rate, ubiquinone levels respond to vitamin A status, it seems necessary to ascertain experimentally whether vitamin K status can also regulate ubiquinone levels in particular tissues.

The simplest method of producing vitamin K deficiency in rats is perhaps the dietary administration of sulphonamides (Black, McKibbin & Elvehjem, 1941; Black, Overman, Elvehjem & Link, 1942; Welch & Wright, 1943). Kornberg, Daft & Sebrell (1944*b*), however, found that on a diet containing 1 % of sulphadiazine neither the caecal contents nor the faeces of rats exhibited vitamin K activity. Sulphamerazine at the 1 %

dietary concentration failed to produce hypoprothrombinaemia in 4 weeks (Mushett & Seeler, 1947). The results of Kornberg, Daft & Sebrell (1944*a*) indicated that sulphadiazine was the most suitable material to administer. In view of the well-known lack of high structural specificity among vitamin K-active substances (Almquist, 1954) and the many unsolved problems of modes of action, the first approach with a dietary sulphadiazine supplement was not by itself adequate or conclusive. Accordingly, a diet was used which lacked vitamin K_1; this could be supplemented by either menadione or sulphadiazine. In this way the vitamin K deprivation would be more stringent and for the controls the replacement would be (in some respects at least) unequivocal. The replacement was effective, but whether the menadione functioned as such or as a precursor for vitamin K_2 was not pursued. The growth-retarding effects of vitamin K deficiency were especially well-marked and the fact that animals which died showed haemorrhagic lesions confirmed the speedy appearance of the deficiency syndrome.

The investigation would also have been incomplete without a study of dicoumarol-fed rats. Dicoumarol may act by injuring the liver or as antagonizing vitamin K by competitive inhibition (see Mentzer & Meunier, 1943; Woolley, 1944, 1945). The idea, advanced by Collentine & Quick (1951), that dicoumarol displaces vitamin K in enzyme systems producing prothrombin, fits the observations of Spinks & Jaques (1950) to the effect that ^{14}C-labelled dicoumarol accumulates mainly in the liver and that its disappearance from the liver is accelerated when vitamin K is administered. The longer the dicoumarol is allowed to remain in the liver the greater its effect in reducing the prothrombin concentration. The mode of action may be a multiple process (cf. Dam, 1948; Sørbye, Kruse & Dam, 1950).

The final step in the present study was to ascertain whether the additional stress of administering sulphadiazine to rats suffering from vitamin A deficiency causes any marked change in the occurrence of ubiquinone, substance SC and other constituents of the tissue unsaponifiable matter.

These tests show that vitamin K status has remarkably little effect on most of the variables under study. Sulphadiazine has a striking effect on the spleen which deserves further study. In the first experiments (see Tables 1 and 2) growth was markedly retarded and organ weights were smaller. In liver, kidney and intestine there was no evidence that vitamin K_2 deficiency had appreciably affected the vitamin A, sterol, ubiquinone, substance SC and hydrocarbons. There was possibly an increase in vitamin A in the liver but this was offset, perhaps, by the reduction in liver weight.

The experiment which aimed at excluding dietary vitamin K_1 as well as inhibiting production of vitamin K_2 (Table 7) also showed striking retardation of growth. Again, the results showed that vitamin K status had little effect on the hydrocarbon, ubiquinone, sterol and substance SF (3-formylindole) contents of liver. In this experiment the decrease in vitamin A content was noteworthy.

The feeding of dicoumarol caused a small but statistically significant (P 0·01) reduction of body weight. The minor constituents of liver listed in Table 8 were not much changed, although some increase in hydrocarbon content was indicated.

The final experiment, with vitamin A-deficient rats, showed how the increase in ubiquinone content of liver which results from avitaminosis A is maintained or even enhanced in avitaminosis A and K but reversed by vitamin A supplementation. In the rehabilitated rats the increased weight of liver tended to exaggerate a decline in ubiquinone content, which was none the less real. Similarly, the animals given vitamin A showed less substance SC than those deprived of vitamin A. The additional stress of vitamin K deprivation neither exacerbated nor mitigated the vitamin A-deficiency syndrome as it affects the minor constituents of rat-liver unsaponifiable matter. Therefore despite the fact that ubiquinone and the K vitamins are quinones and that vitamin K_2 shares with ubiquinone a polyisoprenoid side chain, the present experiments disclosed remarkably little biochemical interaction.

SUMMARY

1. Weanling rats kept for 29 or 36 days on a diet containing 1% of sulphadiazine showed as compared with control rats smaller livers, kidneys, intestines and very much smaller spleens. The prothrombin content of plasma was reduced to about one-fifth. Despite the induced vitamin K_2 deficiency the concentrations of ubiquinone, vitamin A, sterol and hydrocarbon in the tissues were but little affected.

2. Weanling rats kept on a diet free from vitamin K_1 and containing sulphadiazine to inhibit bacterial formation of vitamin K_2 were compared with rats on the vitamin K_1-free diet supplemented with menadione. The vitamin K-deficient rats showed retarded growth and decreased liver vitamin A as well as smaller livers. Liver concentrations of ubiquinone and sterol were about the same in the control rats as in the deficient rats.

3. Weanling rats kept for 26 days on a diet containing dicoumarol (30 mg./kg.) differed from control rats slightly but significantly in body weight. No other signs of vitamin K deficiency were seen.

4. Rats which had been on a vitamin A-deficient diet for 45 days after weaning had exhausted their liver reserves of vitamin A, and the liver concentrations of sterol, ubiquinone and substance SC had all risen. One group of such rats was given curative doses of vitamin A; the survivors showed restored growth and diminutions in liver ubiquinone and substance SC concentrations. A similar group received a sulphadiazine supplement which added to vitamin A deficiency a conditioned vitamin K_2 deficiency. The superimposed vitamin K_2 deficiency did not alleviate nor appreciably exacerbate the effects of vitamin A deficiency on liver concentration of sterol, ubiquinone and substance SC.

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Unsaponifiable Constituents of Liver, Kidney and Heart Tissues from Vitamin E-Deficient Rats Compared with α-Tocopherol-Supplemented Rats

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The tocopherols can be converted *in vivo* into *p*-benzoquinone derivatives such as α-tocopherylquinone (I). This makes it necessary to investigate whether there is any simple relationship between tocopherol status and the ubiquinone (II, for references see Morton, 1958) content of tissues, because (i) the precise roles of vitamin E and ubiquinone are of interest in the study of lipid cofactors and (ii) the origin of the aromatic portion of the ubiquinone molecule has not been established. The biogenesis of the polyisoprenoid side chain in ubiquinones (e.g. the $-C_{50}H_{81}$ side chain in the animal

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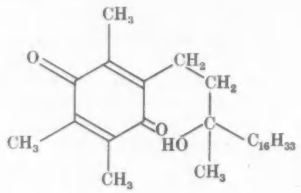
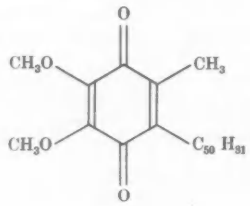
quinone which can be designated ubiquinone 50) proceeds on familiar lines, with mevalonic acid as an intermediate (Gloor & Wiss, 1959). It will be shown that in rats tocopherol status and ubiquinone contents of heart, liver and kidney are unrelated.



(I)



(II)

METHODS

Animals. Weanling hooded rats bred in the Department were used.

Diet. Vitamin E-low diet (based on that of Mason & Harris, 1947) consisted of vitamin-free casein 20%, sucrose 56%, brewer's yeast 10%, lard 10%, salt mixture (Hubbell, Mendel & Wakeman, 1937) 4% and ergocalciferol 1 mg./100 lb. The control animals were given the above diet fortified with α-tocopheryl acetate (10·25 g./100 lb. or 0·215 g./kg.). The diet was also supplemented with β-carotene (3000 i.u./kg.), the aim being to meet the requirement of vitamin A for good growth but not for any considerable liver storage.

Plan of experiment. Sixteen male and 16 female rats were divided into four groups: *A* (eight males) and *B* (eight females) were given the basal diet supplemented with α-tocopheryl acetate; two similar groups, *C* and *D*, were given the vitamin E-deficient diet. The experiment ran for 127 days. The animals were killed with chloroform and the liver, kidneys, heart and gonads were removed.

Evidence of deficiency. The teeth and uteri of the deprived and the supplemented animals were compared (Martin & Moore, 1939; Moore & Wang, 1943, 1947). The incisor teeth of the animals given α-tocopheryl acetate were of the normal yellow–orange colour, whereas those of the deficient animals were white or at most very faintly yellow. All the deprived females showed the characteristic brown colour of the uterus as opposed to the faint pink colour of the normal uterus.

Saponification and chromatography. The tissues were saponified as described by Mervyn & Morton (1959) and chromatographed on acid-washed alumina partially deactivated to Brockman grade 3.

RESULTS

The vitamin E deprivation over a period of 127 days did not greatly influence the rate of growth. The male animals on the deficient diet were on the whole lighter than the controls and the organ weights were smaller (see Table 1). This was most noticeable in the testes, which not only weighed less but accounted for a smaller percentage of total body weight.

Chromatography of the liver unsaponifiable matter from the vitamin E-supplemented animals disclosed the presence in the 2% ether in light petroleum eluate of a substance with twin maxima at 292 and 298 mμ in *cyclo*hexane. The compound responsible (provisionally designated SG) was absent from the corresponding eluate derived from the deprived rats, and it was clearly related to and derived from the α-tocopheryl acetate supplement. The unusual thing about substance SG is that it precedes ubiquinone in the chromatography. Nevertheless, when the crude SG fraction is acetylated the absorption spectrum in *cyclo*hexane shows $\lambda_{max.}$ 278 and 284 mμ with minima at 255 and 282 mμ (α-tocopheryl acetate: $\lambda_{max.}$ 279 and 285 mμ; minima 255 and 283 mμ). It seems probable that SG is α-tocopherol carried through the chromatographic column by a hydrocarbon. In some

Table 1. *Comparison of vitamin E-deficient rats with control animals given α-tocopheryl acetate*

	Mean weight (g.)			
	Vitamin E-supplemented		Vitamin E-deficient	
	Males	Females	Males	Females
Body (Initial)	42	45·5	37·5	43·5
Body (Final)	260	189	229	183
Liver	10·9	8·5	8·75	8·4
Kidney (pair)	2·11	1·61	1·87	1·58
Heart	0·83	0·63	0·73	0·63
Testes	2·58	—	1·81	—
Uterus	—	0·35	—	0·38
Testes (% of body wt.)	0·99	—	0·79	—
Uterus (% of body wt.)	—	0·185	—	0·207

fractions eluted by 20 % ether in light petroleum a small amount of α-tocopherylquinone was found ($\lambda_{max.}$ 260 and 270 mμ). This fraction also contained cholesterol and 7-dehydrocholesterol. When it was acetylated and rechromatographed the esterified sterols were readily eluted by light petroleum containing a little ether, whereas the α-tocopherylquinone was eluted only by ether itself. Liver storage of vitamin A was either very small or negligible. Substance SC (Lowe, Morton & Harrison, 1953) was not found in more than trace amounts.

Table 2 indicates that the vitamin E-deficient animals were very similar to the control animals in the hydrocarbon, ubiquinone and sterol contents of liver unsaponifiable matter. The occurrence of SG (tocopherol?) and α-tocopherylquinone in the supplemented animals but not in the deprived animals confirms the efficacy of the dietary regimen. The sexes behaved similarly. Table 2 also shows how the kidney and heart tissues showed little difference between the supplemented and deprived animals in hydrocarbon, sterol and ubiquinone content. The analytical work on the testes of the deprived animals was marred by incomplete saponification; there can, however, be no doubt that neither the ubiquinone nor the sterol contents were appreciably different from those for normal testes.

DISCUSSION

In rats the heart tissue is richer in ubiquinone than the liver or kidney tissue. Deprivation of vitamin E for 127 days made no demonstrable difference to the ubiquinone contents. Heaton, Lowe & Morton (1957) did not detect ubiquinone in rat testes; the present work shows that although the amount is not large ubiquinone is present. There is about 1·2 mg. of ubiquinone in the entire body of the weanling rat; this is less than the amount found in a few selected tissues of the vitamin E-deprived rat 127 days later. It therefore seems that α-tocopherol is neither a precursor of ubiquinone nor is it needed in any enzymic step in the biosynthesis of ubiquinone. The substance SC was previously (Lowe *et al.* 1953; Heaton *et al.* 1957) found to increase in amount in the livers of vitamin A-deficient rats. The substance SC content of the liver of the vitamin E-deprived rats and of the tocopheryl acetate-supplemented controls was negligibly small in both cases.

Table 2. *Comparison of unsaponifiable constituents from tissues of vitamin E-deficient rats with those from control animals given α-tocopheryl acetate*

	Vitamin E-supplemented		Vitamin E-deficient	
	Males	Females	Males	Females
Liver				
Hydrocarbon (mg./g.)	0·12	0·19	0·19	0·11
Ubiquinone (μg./g.)	117	114	110	138
Sterol (mg./g.)	2·07	1·93	2·49	2·05
Substance SG (units/g.)*	3·1	4·1	Nil	Nil
Tocopherylquinone (μg./g.)	4·1	6·3	Nil	Nil
Kidney				
Unsaponifiable (%)	0·62		0·64	
Hydrocarbon (μg./g.)	61		74	
Ubiquinone (μg./g.)	108		118	
Sterol (mg./g.)	3·83		4·03	
(Vitamin A, 7-dehydrocholesterol and 3-formylindole were detected in both batches)				
Tocopherylquinone	Present		Not detected	
Heart				
Unsaponifiable (%)	0·36		0·27	
Hydrocarbon (mg./g.)	0·35		0·14	
Ubiquinone (μg./g.)	233		224	
Sterol (mg./g.)	1·50		1·34	
7-Dehydrocholesterol	Detected		Detected	
Tocopherylquinone	Trace		Not detected	
Testes				
Unsaponifiable (%)	0·28		0·73	
Hydrocarbon (mg./g.)	0·07		Doubtful	
Ubiquinone (μg./g.)	25		$< 18 > 30$	
Sterol (mg./g.)	1·6		2·06	
3-Formylindole	Detected		Detected	
Tocopherylquinone	Trace		Not detected	
7-Dehydrocholesterol	Not detected		Not detected	

* Units represent $E^{1\%}_{1\,cm.}$ 298 mμ for the SG fraction multiplied by its weight in mg.

The material SG obtained from the tissues of the tocopheryl acetate-supplemented rats is slightly less strongly adsorbed than ubiquinone; it is very similar to α-tocopherol but it may be a metabolite with a slightly changed side chain. The tocopherylquinone found in the controls might be a 'detoxication' intermediate or a part of the tocopherylquinol redox system.

The effects of vitamin E deficiency on sterol content and hydrocarbon content are unimportant. In considering the results as a whole it seems probable that the biological role or roles of tocopherols are not directly concerned with other unsaponifiable constituents of tissue lipids.

The control rats in the present experiments obtained in the diet about 2·0–2·5 mg. of α-tocopheryl acetate/day. This is at least twice the minimum requirement (Deuel, 1957). Mason (1940) found that in the male rat prevention of irreversible damage to the testes makes it obligatory to provide the vitamin from the age of 40–50 days, whereas the requirement of female rats is not critical until after conception and implantation. In the present experiments the male rats were more responsive than the females to vitamin E deprivation (Table 1), but with respect to liver ubiquinone and sterol both sexes were equally unresponsive.

SUMMARY

1. Two groups (males and females) of weanling rats were given a vitamin E-free basal diet for 127 days with a tocopherol supplement. Two similar groups were given the unsupplemented diet and when killed were vitamin E-deficient.

2. The unsaponifiable fractions from liver, heart and kidney were chromatographed on alumina. Ubiquinone contents of approx. 120, 110, 230 and 25 μg./g. were found for liver, kidney, heart and testes.

3. The vitamin E status had no striking influence on the amounts of hydrocarbon, sterol or ubiquinone obtained from the different tissues.

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Effects of Vitamin A Deficiency and Adrenalectomy on Ubiquinone and Substance SC in Rat Liver

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Lowe, Morton & Harrison (1953) described changes in the adrenal glands of rats suffering from vitamin A deficiency. In the normal rat the adrenals contain very little vitamin A (Moore, 1957). Bailly & Nettor (1932) found β-carotene in the cortex of ox adrenals but not in the medulla. Davies & Moore (1934) observed that rats given large (but not toxic) doses of vitamin A esters were capable of storing relatively large amounts of vitamin in the adrenal glands (1500 i.u./g. of adrenal tissue). Popper & Greenberg (1941), by fluorescence microscopy, found that the vitamin A of adrenal glands was sharply localized in the zona fasciculata even when the animals were hypervitaminotic.

Bennet, Bennet, Shaver & Gracht (1950) exposed the whole bodies of rats to X-irradiation and found absorption of vitamin A alcohol to be apparently unaffected, but 6 hr. after giving the vitamin the adrenals of the control animals contained more vitamin A than those of the irradiated animals. After 10 hr. however, the latter contained more than the former.

Willmer & Laughland (1957) administered β-[^{14}C]carotene to rats and found that the concentration of radioactivity became highest in the adrenals, followed in decreasing order by the

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pituitary gland and the liver. The adrenal lipid had 91·8 % of its potential vitamin A activity as preformed vitamin A and only 3·8 % as provitamin A (β-carotene). Lowe *et al.* (1953) observed that in vitamin A deficiency the phospholipid content of rat adrenals was reduced and the histological results indicated that the residual phospholipid was confined to the zona glomerulosa. The disappearance of phospholipid from the rat zona fasciculata may be significant since this zone is often thought to be the site of glucocorticoid formation. Wolf, Lane & Johnson (1957) and Wolf, Wagle, van Dyke & Johnson (1958) showed that vitamin A deficiency impaired the synthesis of glucocorticoid hormones. It could therefore be argued that vitamin A deficiency led to partial 'chemical adrenalectomy' with diminished glucocorticoid formation (or secretion) and consequent lowering of glyconeogenesis.

Clark & Colburn (1955) found that the administration of cortisone to normal or adrenalectomized rats brought about a rapid depletion of the stores of vitamin A in liver and kidney. The stress of semi-starvation also resulted in a decline in the liver stores of vitamin A, but the effect was less than that caused by cortisone. The hyperactivity of the adrenals under stress thus played a part in the loss of liver vitamin A. Bodansky & Markardt (1951) observed that Reichstein's compound L reduced the concentration of vitamin A in rat blood. Adrenaline did not have this effect.

The foregoing considerations suffice to show that vitamin A deficiency influences adrenal function. Heaton, Lowe & Morton (1957) described how vitamin A deficiency brought about marked increases in substance SA (ubiquinone) and substance SC contents of rat liver. Morton & Phillips (1959*a*) have confirmed this and have shown that the ubiquinone content rises steadily as the deficiency develops. The rise in substance SC is a late manifestation of deficiency. It was therefore desirable to investigate whether any changes in tissue ubiquinone levels occur in the normal and vitamin A-deficient rat after adrenalectomy.

METHODS

Animals. Hooded or albino rats reared in the University were used.

Diets. Two vitamin A-deficient diets were used. (*a*) Casein (vitamin-free) 18 %, starch 65 %, arachis oil 5 %, salt mixture (U.S.P.XIII) 4 %, Marmite 8 %. A vitamin supplement (Morton & Phillips, 1959*b*) was added (2 g./kg. of diet). (*b*) Casein (vitamin-free) 18 %, sucrose 65 %, dried brewer's yeast 8 %, arachis oil 5 %, salt mixture 4 %. In experiments with stock-colony animals Diet no. 4 (British Extracting Co., Bromborough, Cheshire) was used.

Adrenalectomy. Both adrenals in each rat were removed, under ether anaesthesia, in the customary manner through a midline dorsal skin incision. We are greatly indebted to Professor R. G. Harrison of the Department of Anatomy, who performed all the operations.

Examination of liver tissue. The liver was saponified (in the presence of ethanolic pyrogallol) and the unsaponifiable fraction was chromatographed on alumina partially deactivated to Brockmann grade 3 as described by Mervyn & Morton (1959). The first eluate, obtained with light petroleum alone, contained a small amount of hydrocarbon and exhibited an inflexion at 260 mμ. Ubiquinone was eluted in the fractions obtained with 4–6 % of ether in light petroleum (b.p. 40–60°). The main sterol fractions, eluted by 20 % of ether in light petroleum, were examined for ultraviolet absorption and then acetylated and rechromatographed.

The acetylated sterol fractions consisted in the main of cholesteryl acetate, which was readily eluted by 1 % of ether in light petroleum. A small amount of material (about 1 % of the acetylated fraction) was eluted by 2 % of ether in light petroleum; it showed $\lambda_{max.}$ 280 mμ in *cyclo*hexane, $E^{1\%}_{1\,cm.}$ approx. 17. Substance SC acetate was eluted by 8 % of ether in light petroleum and showed the characteristic absorption peaks at 275 and 315 mμ and the inflexion at 285 ($E^{1\%}_{1\,cm.}$ 275 mμ 57). Ether alone eluted free substance SC ($\lambda_{max.}$ 275 mμ with inflexions at 285 and 330 mμ).

Experiment 1. Fourteen female rats were kept on the vitamin A-deficient diet (*a*) for 45 days from the time of weaning. Seven of the rats were bilaterally adrenalectomized on the forty-fifth day and were then given 0·9 % sodium chloride to drink instead of water. The vitamin A-deficient diet was offered to these animals and to the controls during the next 43 hr. All the animals were then killed (chloroform) and the livers and kidneys were excised and retained for analysis. By the forty-fifth day all 14 animals were in an advanced state of vitamin A deficiency. Three of the adrenalectomized animals died during the last 17 of the 43 hr. after operation, but their livers were included in the material tested.

Experiment 2. Ten male and ten female albino rats were placed at, or very soon after, weaning on the vitamin A-deficient diet (*b*). In this experiment the aim was to carry out the adrenalectomy after the vitamin A reserves had been almost exhausted but before the deficiency syndrome had become far advanced. By the thirty-third day of experiment growth had slackened or ceased and most of the animals had reached the stage of the weight plateau. Half the animals were then bilaterally adrenalectomized. One operated animal and one control animal died on the third day and their tissues were not used. The 18 remaining animals were killed 72 hr. after the operations. Livers were excised and retained for analysis.

Experiment 3. Twenty male and female rats were given the stock diet from weaning until they were 57 days old. Five of each sex were bilaterally adrenalectomized and maintained for a further 10 days on the stock diet. All the animals were killed and the livers excised on the tenth post-operative day.

RESULTS

Experiment 1. Table 1 summarizes the results. Both the control animals and the adrenalectomized animals were frankly avitaminotic and the post-operative period was short. The hydrocarbon

content of the liver was above the normal value. The ubiquinone content of the livers of the control animals was at least four times the normal amount. In the livers of the adrenalectomized animals it was about three times the normal, but more work is needed to test whether the difference between three times and four times the normal is other than fortuitous. In this experiment the livers from the adrenalectomized animals which died in the later part of the post-operative period were included with those from surviving animals. Because of this the ubiquinone content for the group may have erred on the low side. Perhaps the important point is that the ubiquinone content was at least three times the normal amount. The substance SC content of the liver from both groups was also well above the normal but the superimposition on vitamin A deficiency of the consequences of adrenalectomy did not change the situation. The total liver sterol was, however, raised by about 23%.

Experiment 2. In this trial both sexes were studied. The animals had used up their stores of vitamin A and were not yet losing weight. They had no xerophthalmia. Table 2 summarizes the results. The sharp increase in the hydrocarbon content of liver which was seen in experiment 1 had not yet occurred. The ubiquinone content of the livers of the deprived animals was about twice, and of the deprived and adrenalectomized animals about thrice, the normal amount. This was true for both sexes. The substance SC fraction for the female adrenalectomized group was lost but the figures for males in Table 2 showed that the rise in substance SC was only incipient. The sterol content was higher in the adrenalectomized animals. The broad picture is that in rats about to become frankly avitaminotic, adrenalectomy brought the animals nearer, in respect of the criteria considered here, to the very seriously affected animals of Expt. 1. The marked loss of liver weight in the adrenalectomized rats is noteworthy.

Experiment 3. In this experiment all the animals had ample liver stores of vitamin A. Both sexes were studied and all the adrenalectomized animals were alive 10 days after operation. During this period of 10 days the control animals had increased in weight (males by 33 g., females by 21 g.), whereas the adrenalectomized animals remained practically stationary in weight. Table 3 summarizes the results. The control rats had larger livers (expressed as a percentage of body weight) than the adrenalectomized rats. The hydrocarbon content was low

Table 1. *Comparison of vitamin A-deficient rats with adrenalectomized vitamin A-deficient rats*

Seven female hooded rats exhibiting advanced avitaminosis A were in each group.

	Vitamin A-deficient	Adrenalectomized vitamin A-deficient
Mean body wt. day 45 after weaning (g.)	81	81
Mean liver wt. (g.)	3·61	3·59
Liver constituents		
Unsaponifiable matter (%)	0·654	0·632
Hydrocarbon (mg./g.)	0·86	0·85
Vitamin A	Not detected	
Ubiquinone (μg./g.)	511	397
Substance SC (μg./g.)	110	105
Sterol (mg./g.)	2·93	3·61

Table 2. *Comparison of vitamin A-deprived rats with adrenalectomized vitamin A-deprived rats*

Animals of both sexes were at or near the growth plateau.

	Vitamin A-deprived		Vitamin A-deprived and adrenalectomized	
Sex	Males	Females	Males	Females
No.	4	5	5	4
Mean final body wt. (g.)	100·5	103·2	101·2	92
Mean liver wt. (g.)	4·73	4·88	3·59	3·89
Liver constituents				
Unsaponifiable matter (%)	0·438	0·405	0·459	0·521
Hydrocarbon (mg./g.)	0·22	0·08	0·04	0·12
Vitamin A	Not detected			
Ubiquinone (μg./g.)	222	212	356	365
Substance SC (μg./g.)	55	44	70	Lost
Sterol (mg./g.)	2·68	2·15	3·06	3·46

Table 3. *Comparison of stock-colony rats on a normal diet with adrenalectomized rats on a normal diet*

Adrenalectomy occurred at 57 days of age; all animals were killed 10 days later. The relatively high vitamin A content of liver causes interference with determination of substance SC but there was evidence of no more than trace amounts of substance SC.

	Control		Adrenalectomized	
	Males	Females	Males	Females
Mean initial wt. (g.)	179	124	180	123
Mean final wt. (g.)	212	145	183	127
Mean liver wt. (g.)	12·84	8·78	8·74	6·67
Liver (% of body wt.)	6·06	6·05	4·78	4·78
Liver constituents				
Hydrocarbon (mg./g.)	0·072	0·098	0·089	0·098
Vitamin A (i.u./g.)	151	164	137	216
Ubiquinone (μg./g.)	110	119	133	130
Sterol (mg./g.)	1·80	2·13	2·23	2·12

throughout. The ubiquinone content rose a little (expressed as μg./g. of liver) in the adrenalectomized animals, but expressed as μg./liver it fell a little. This indicates that the ubiquinone content of rat liver was very little affected as a result of adrenalectomy when vitamin A reserves were substantial. The sterol content of liver was also little changed.

DISCUSSION

Cowie (1949) showed that completely adrenalectomized rats could survive from 8 to 19 days, depending upon their age at operation. All the animals in Expt. 3 survived for 10 days and some might have lived longer. We do not think that the experiment was vitiated by the development of 'accessory' adrenal tissue. Vitamin A deficiency shortened the survival time of adrenalectomized animals. Adrenalectomy hastened the appearance of some of the late manifestations of vitamin A deficiency in animals having no remaining liver store. If, however, the pre-operative deficiency was very advanced the further results of adrenalectomy were much less striking. The third experiment suggests that the rises in ubiquinone and substance SC concentration which occur in vitamin A deficiency are not caused by the partial 'chemical' adrenalectomy of deficiency since total adrenalectomy (surgical) of animals normal in vitamin A status leaves ubiquinone and substance SC at practically normal concentrations in liver. The rise observed by Heaton *et al.* (1957) and confirmed by Morton & Phillips (1959*a*) is seen even more clearly to reflect vitamin A status in the rat.

SUMMARY

1. The rises in ubiquinone and substance concentration SC which occur in the livers of vitamin A-deficient rats may be hastened if at an early stage in the deficiency the rats are bilaterally adrenalectomized.

2. If, however, the deficiency syndrome is already far advanced adrenalectomy does not noticeably influence the concentrations of minor constituents of liver unsaponifiable matter.

3. In rats on a stock diet adrenalectomy brought about no striking changes in liver vitamin A (range 137–216 i.u./g. of liver), ubiquinone content (range 110–133 μg./g.) or sterol content (range 1·80–2·23 mg./g.). Substance SC was not present in more than trace amounts.

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The Inactivation of Ribonuclease during the Isolation of Ribonucleic Acids and Ribonucleoproteins from Yeast

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The isolation of ribonucleic acids and ribonucleoproteins in an undegraded state is complicated by the presence of degradative enzymes, particularly ribonuclease. It has been particularly difficult to find an inhibitor for this enzyme that was suitable for use in preparative work on the isolation of ribonucleoproteins. Inhibitors such as sodium cholate (Jones, Marsh & Rizvi, 1957) and sodium dodecyl sulphate (Crestfield, Smith & Allen, 1955), which have been used for the isolation of ribonucleic acids, cannot be used for the isolation of ribonucleoproteins because of their possible denaturing action on the proteins. Polyacidic substances have been used to inhibit pancreatic ribonuclease (Zöllner & Fellig, 1953; Vandendriessche, 1956) and, although these could possibly be used in the isolation of ribonucleoproteins, the removal of the inhibitor from the final product would be difficult. Copper and Zn^{2+} ions inhibit pancreatic ribonuclease (Zittle, 1946; Davis & Allen, 1955) but, as the present investigations have shown, these have limited use in isolation procedures for ribonucleic acids and ribonucleoproteins. The object of the present work was to find inhibitors suitable for this purpose.

EXPERIMENTAL

Nitrogen was determined by Belcher & Bhatty's (1956) method and phosphorus as described by Jones, Lee & Peacocke (1951). Protein (5–100 μg.) was estimated by the use of the Folin–Ciocalteu reagent as described by Lowry, Rosebrough, Farr & Randall (1951). Crystalline ovalbumin was used as the standard. Analyses were performed on moist material and corrected for moisture contents determined on separate samples.

Estimation of nucleic acid-hydrolysing enzymes. The simple and rapid method described by Kunitz (1946) for the estimation of ribonuclease activity, in which the decrease in extinction at 290 mμ of the ribonucleic acid (RNA) substrate is measured without the necessity of precipitating the undegraded nucleic acid, could not be used in this case because pure substrates were not being used; the estimations were carried out on the crude yeast extracts without the addition of more substrate. This gave a measure of the amount of degradation actually occurring in the extract. The following procedure was adopted for the estimation of this degradation.

The suitably diluted yeast extract in acetate buffer (0·01 M-sodium acetate–0·64 mM-acetic acid), pH 6·0, I 0·01, was incubated at 37° in the presence of a few drops of toluene. At intervals samples (1 ml.) were removed and mixed with ethanolic 0·3 M-zinc chloride (0·5 ml.). After 10 min. the solutions were centrifuged at 3000 rev./min. for 3 min. A sample (1 ml.) of the supernatant liquid was removed, diluted five times and the extinction at 260 mμ determined. The zinc chloride–ethanol reagent gave more consistent results than a hydrochloric acid–ethanol reagent and, unlike calcium chloride–ethanol (Jones *et al.* 1957), it could be used in the presence of ammonium sulphate. Determinations were carried out at I 0·01, 0·1, 1·0 and 3·0. With the last two the ionic strength was maintained by means of ammonium sulphate. The effect of 0·6 mM-Zn^{2+} ions on the activities at these various ionic strengths was also studied. The results are shown in Fig. 1.

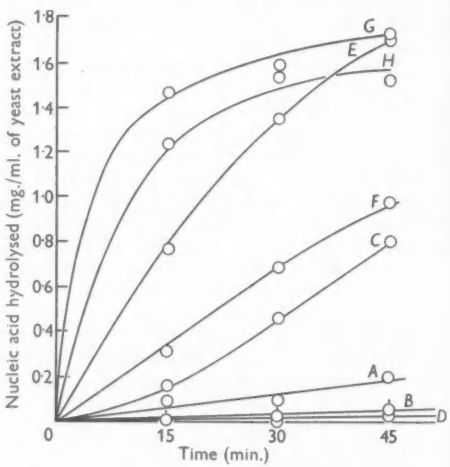

Fig. 1. Enzymic hydrolysis of nucleic acids in extracts of disintegrated yeast at 37°. *A* and *B*, I 0·01; *C* and *D*, I 0·10; *E* and *F*, I 1·0; *G* and *H*, I 3·0. *B*, *D*, *F* and *G* contained 0·6 mM-Zn^{2+} ions. The amount of nucleic acid hydrolysed was calculated from the extinction at 260 mμ on the basis that a solution of RNA containing 50 μg./ml. has E 1·0. It was found that the small contribution of other ultraviolet-absorbing components could be neglected in this instance.

Preparation of yeast extracts. A suspension of baker's yeast [120 g. of moist pressed yeast (The Distillers Co. Ltd.) equivalent to 30 g. of freeze-dried yeast] in water (250 ml.) was adjusted to pH 7·5 with sodium bicarbonate solution and shaken in a vibratory ball mill (Griffin and George Ltd.) at 0° with an equal volume of Ballotini glass beads (1 mm. diam.). From time to time the pH was readjusted to 7·5 with sodium bicarbonate solution. When almost complete breakage of the cells had been achieved ($2\frac{1}{2}$–3 hr.), the liquid was decanted from the glass beads, the latter were washed with water and cell debris was removed from the combined liquid and washings by centrifuging at 20 000 *g*. The supernatant liquid was dialysed against acetate buffer, pH 6·0, *I* 0·01.

Preparation of a standard bentonite suspension. A suspension of bentonite (10 g.; technical grade, British Drug Houses Ltd.) in water (200 ml.) was prepared by thorough stirring and the coarse particles were removed by centrifuging at 2600 rev./min. for 15 min. The fine clay suspension was decanted off and centrifuged at 9000 *g* for 20 min. The cloudy supernatant liquid was discarded and the sediment suspended in acetate buffer, pH 6·0, *I* 0·01. After homogenization in the Ato-Mix blendor (Measuring and Scientific Equipment Ltd.) the suspension was centrifuged at 9000 *g* and the supernatant liquid discarded. This process of suspension in acetate buffer and centrifuging (now at 20 000 *g*) was repeated until the extinction at 260 mμ of the supernatant liquid in a 1 cm. cell had decreased to about 0·7. The bentonite was finally suspended in the acetate buffer (50 ml.).

Adsorption of the nucleic acid-hydrolysing enzymes by bentonite. Samples (10 ml.) of the yeast extract in acetate buffer, pH 6·0, *I* 0·01, were mixed with various volumes of the bentonite suspension. After shaking the suspensions at 0° for 17 hr. the bentonite was removed by centrifuging at 9000 *g*. The enzymic degradation occurring at 37° in the supernatant liquids was determined after increasing the ionic strength to 2·4 by the addition of ammonium sulphate, and the ultraviolet-absorption spectra of the supernatant liquids were also measured. The results are shown in Fig. 2.

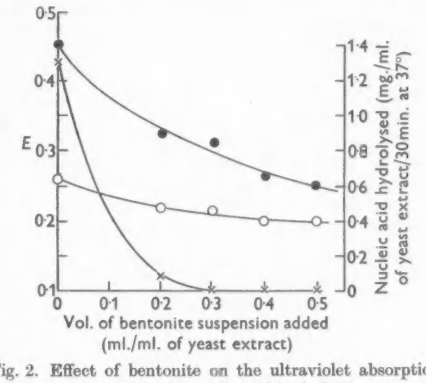

Fig. 2. Effect of bentonite on the ultraviolet absorption and on the activity of nucleic acid-hydrolysing enzymes in an extract of disintegrated baker's yeast in acetate buffer, pH 6·0, *I* 0·01. ●, $E_{220\,m\mu}$; ○, $E_{260\,m\mu}$; ×, enzymic hydrolysis of nucleic acids.

Isolation of ribonucleic acids and ribonucleoproteins

An extract of yeast in acetate buffer, pH 6·0, *I* 0·01, containing 0·6 mM-Zn^{2+} ions (prepared from 225 g. of moist, pressed yeast as previously described except that the yeast was broken in the presence of 0·6 mM-Zn^{2+} ions) was shaken for 18 hr. at 0° with standard bentonite suspension (420 ml.) and the bentonite then removed by centrifuging at 20 000 *g*. After correction for dilution, the extinction of the supernatant liquid at 260 mμ had been reduced 24% by the action of the bentonite. The bentonite-treated solution was adjusted to an ethanol concentration of 20% by the addition of ethanol–acetate buffer, pH 6·0, *I* 0·05 (containing 3 mM-zinc acetate) (4:1) previously cooled to −10°. The zinc concentration was increased to 0·01 M and the temperature maintained at 0°. The material so precipitated was centrifuged off and dissolved in acetate buffer, pH 6·0, *I* 0·01, containing 0·6 mM-Zn^{2+} ions, and dialysed against the same buffer for 18 hr. The zinc and ethanol fractionation was repeated, 96% of the material being precipitated at a Zn^{2+} ion concentration of 0·8 mM in 20% ethanol at pH 6·0 and at 0°. The precipitated nucleoprotein was dissolved in water and a portion of the solution dialysed against distilled water and freeze-dried to give the nucleoprotein YNP [102 mg.; nitrogen, 12·1%, phosphorus, 6·3%; nitrogen/phosphorus (w/w) 1·92; protein, 5·0%]. The remainder of the solution was fractionated with ammonium sulphate, 80% of the material being precipitated at 25% (w/v) of ammonium sulphate. This fractionation was repeated twice. The precipitate was finally dissolved in water and exhaustively dialysed against distilled water. There was, however, still present inorganic material, probably from the bentonite, so the nucleic acid was precipitated with cetyltrimethylammonium bromide (Jones, 1953). The resulting precipitate was dissolved in

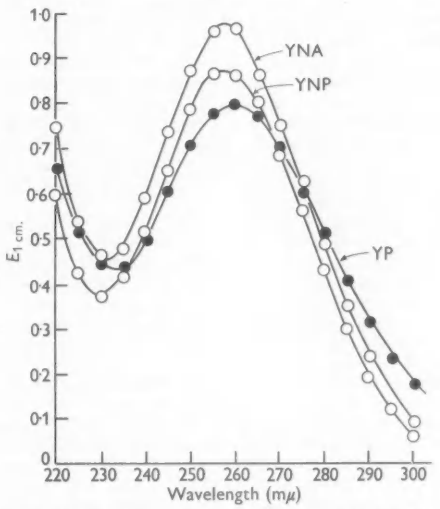

Fig. 3. Ultraviolet-absorption spectra in water of RNA-containing fractions of yeast (50 μg./ml.).

Table 1. *Ribonucleotide content of ribonucleic acid-containing fractions from yeast*

Fraction	Mol. of ribonucleotide per 100 mol. of nucleotides: Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Recovery of ribonucleotides (% based on phosphorus)
YNP	26·3	27·5	20·2	25·9	90
YNA	28·3	26·1	21·0	24·6	99
YP	25·7	24·9	22·6	26·8	92

M-sodium chloride, the nucleic acid precipitated by the addition of ethanol, the precipitate was dissolved in water, dialysed and freeze-dried [YNA; 167 mg.; nitrogen, 16·7%; phosphorus, 9·4%; nitrogen/phosphorus (w/w) 1·76; protein, 2·0%]. The combined supernatant liquids from the three ammonium sulphate fractionations were combined, dialysed and freeze-dried to give YP [25 mg.; nitrogen, 16·1%; phosphorus, 6·7%; nitrogen/phosphorus (w/w) 2·4; protein, 15%]. The three fractions contained no deoxyribonucleic acid (Dische, 1930), no ammonia and only traces of Zn^{2+} ions.

An indication that these methods were successful in inhibiting the ribonuclease was that the ammonium sulphate fractionations were reproducible and no nucleic acid was lost when removing the ammonium sulphate by dialysis. The yield of RNA-containing fractions represented, after making allowance for material used for analysis during the fractionation procedures, about 20% of that originally contained in the organisms. The losses were due to incomplete extraction from the organisms and to the fact that, in order to obtain a pure product, nucleic acid-containing fractions were rejected at various stages in the purification.

The three fractions, which had typical ultraviolet-absorption spectra (Fig. 3), were analysed for ribonucleotides by Crosbie, Smellie & Davidson's (1953) method as modified by Jones, Rizvi & Stacey (1958). The results are shown in Table 1.

Electrophoresis. YNP and YNA were subjected to boundary electrophoresis in the Antweiler micro-electrophoresis apparatus. A 2·5% solution of the sample was used in each case. The buffers used were: citrate, pH 3·5, *I* 0·02; phosphate, pH 7·7, *I* 0·1; borate, pH 9, *I* 0·1. The runs were carried out for 15 min. at 80 V. The currents were about 0·54, 1·5 and 2·1 mA for the citrate, phosphate and borate buffers respectively.

Fractions YNP and YNA showed only one peak at pH 7·7 and 9·0. At pH 3·5, YNA showed one rapidly moving component and YNP showed two components, the faster one comprising about 75% of the total.

Amino acid composition of the nucleoprotein YNP. YNP (5 mg.) was treated with crystalline ribonuclease (0·01 mg., free from protease) in acetate buffer, pH 6, *I* 0·01 (5 ml.), at 37° for 18 hr. and the digest was exhaustively dialysed. The nucleic acid content of the non-diffusible fraction was thereby reduced by about 60%. The material remaining inside the bag was hydrolysed by boiling with 6N-hydrochloric acid for 16 hr. The neutralized hydrolysate was subjected to two-dimensional chromatography on Whatman no. 1 paper with methanol–water–pyridine (80:20:4, by vol.) and 2-methylpropan-2-ol–butan-2-one–diethylamine–water (40:40:4:20, by vol.) as the solvents (Redfield, 1953).

RESULTS

Properties of the nucleic acid-hydrolysing enzymes of yeast. The presence of these enzymes in the yeast extracts was indicated when attempts to purify yeast nucleoproteins by ammonium sulphate precipitation at 0° resulted in loss of the majority of the material on subsequent dialysis. A study of the enzymic activity of yeast extracts showed (Fig. 1) that at *I* 0·01 and at 37° some degradation of the nucleic acids was occurring. An increase in ionic strength resulted in increased degradation. This degradation was almost completely inhibited by 0·6 mM-Zn^{2+} ions at *I* 0·01 and 0·1 but only slightly inhibited by this concentration of Zn^{2+} ions in the presence of ammonium sulphate at *I* 1·0 and 3·0. Other experiments were carried out in which higher concentrations of zinc were used, but in no case was complete inhibition of the enzymes achieved at high ionic strengths. Other substances were tried as inhibitors, e.g. *p*-chloromercuribenzoic acid, *o*-iodosobenzoic acid, sodium arsenite and sodium fluoride. These were either without effect or showed insufficient inhibition. Copper sulphate precipitated the yeast nucleoproteins but, upon redissolving them, the hydrolytic enzymes were found to be still present. The degradation of the nucleic acids must have been due mainly to a ribonuclease or a non-specific phosphodiesterase, because there was very little deoxyribonucleic acid present in the yeast extracts and the other enzyme capable of degrading RNA, namely polyribonucleotide phosphorylase (Ochoa, 1957), requires the presence of phosphate. Since the behaviour of this enzyme system shows some similarities to pancreatic ribonuclease the enzyme will be called a ribonuclease, although there is no evidence that its specificity is the same as that of pancreatic ribonuclease.

Preferential adsorption of the yeast ribonuclease. A number of adsorbents were tried; charcoal adsorbed the enzymes but adsorbed RNA and ribonucleoprotein as well, and Zeo-Karb 225 and 226 (cf. Hirs, Moore & Stein, 1953) did not adsorb the enzymes sufficiently strongly. The clay, bentonite, which has been used to purify lysozyme (Alderton, Ward & Feveld, 1945), was able, however, to adsorb the ribonuclease from yeast extracts

(Fig. 2). The extinction of the extracts at 260 mμ had been reduced only 16% by the bentonite treatment, showing that little ribonucleoprotein had been adsorbed. The extinction at 220 mμ was reduced much more than that at 260 mμ. Essentially the same results were obtained by using a yeast extract prepared by disintegrating yeast in 0·6 mM-Zn^{2+} ions so as to inhibit the ribonuclease in the initial stages of any isolation procedure for RNA or ribonucleoprotein. The treatment with bentonite also removed proteolytic enzymes.

Properties of the ribonucleic acid and ribonucleoprotein. The nitrogen and phosphorus analyses and ultraviolet-absorption spectra showed that the fractions isolated were complexes of nucleic acid and protein. The relatively low values for YNP were due to the presence of inorganic material which was removed by subsequent treatment with cetyltrimethylammonium bromide. The negative reactions with the Dische test for 2-deoxy-D-ribose and the identification and estimation of ribonucleotides in the alkaline digests of the fractions showed that the nucleic acid was RNA.

There was a discrepancy between the results for the protein contents determined from nitrogen analyses and those determined by using the Folin–Ciocalteu reagent. This can be explained by the fact that different proteins give different colour intensities with this reagent and therefore the results are not absolute. Multiplication of the results by 1·5 gives good agreement in all three cases with those calculated from nitrogen analyses.

The main difficulty in determining the amino acid composition of the products was their low protein contents. The RNA content could be reduced by 60% by the action of ribonuclease but the remainder seemed resistant to the action of the enzyme. Glutamic acid, aspartic acid, cystine and glycine (possibly from the decomposition of purines) were detected in hydrolysates. Other amino acids were present but they could not be definitely identified owing to disturbance of the chromatograms, probably by the products of hydrolysis of the RNA.

DISCUSSION

The properties of the yeast ribonuclease showed similarities to those of pancreatic ribonuclease in the effect of Zn^{2+} ions and low ionic strength (Zittle, 1946; Davis & Allen, 1955). Zinc ions could be used to inhibit the yeast ribonuclease at I about 0·1 but not at higher ionic strengths. The removal of the ribonuclease activity from the yeast extracts by bentonite was probably due to adsorption of the enzyme. This conclusion appears to be justified in that no enzyme activity could be detected in the bentonite-treated extracts even after the addition of ammonium sulphate and although sufficient substrate was present. Large amounts of protein were adsorbed on the bentonite, and in similar work on the ribonuclease of *Klebsiella aerogenes* (C. R. Bayley & A. S. Jones, unpublished work) the ribonuclease could be eluted off the bentonite. It is well known that certain enzymes are readily adsorbed on clays, e.g. trypsin and chymotrypsin on kaolinite (McLaren, 1954), and lysozyme on bentonite (Alderton *et al.* 1945). It was not surprising therefore that the proteolytic enzymes of yeast were adsorbed also. Thus the consecutive use of Zn^{2+} ions and bentonite adsorption provides for the first time a procedure in which RNA and ribonucleoprotein can be isolated from yeast under very mild conditions of temperature and pH and without enzymic degradation of the RNA.

The ribonucleoprotein that was isolated from the bentonite-treated extracts by zinc and ethanol fractionation was remarkable for its low protein content (5–7%). This may have been due to the bentonite treatment because material isolated in other experiments, without this treatment, contained at a similar stage in the isolation procedure about 60% of protein. At present it is impossible to say whether bentonite was acting as a deproteinizing agent and breaking weak nucleic acid–protein bonds or whether the material with high protein content was an artifact caused by association of protein and nucleoprotein. A deoxyribonucleoprotein containing only 9·6% of protein has been isolated from bovine tubercle bacilli (Tsumita & Chargaff, 1958).

Fractional precipitation of the nucleoprotein with ammonium sulphate did not completely dissociate the protein from the nucleic acid. This indicated the presence of linkages other than salt linkages. The RNA (YNA, 2–3% of protein) was titrated by Dr A. R. Peacocke, who found a small hysteresis loop in the acid region similar to that found with the RNA of *Klebsiella aerogenes* (Cox, Jones, Marsh & Peacocke, 1956; Jones & Peacocke, 1957). This could be attributed to hydrogen bonds but there was no evidence that these bonds linked the nucleic acid to the small amount of protein present in the sample. Strong evidence for the presence of hydrogen bonds in a ribonucleoprotein isolated from *Escherichia coli* has been obtained by Elson (1958).

SUMMARY

1. Nucleic acid-hydrolysing enzymes, which are present in yeast extracts, were activated by increasing ionic strength. They were inhibited by 0·6 mM-Zn^{2+} ions at $I \leqslant 0{\cdot}1$ but were not completely inhibited by this and higher concentrations of Zn^{2+} ions at higher ionic strengths. Most of the activity was probably due to a ribonuclease.

2. The ribonuclease and also proteolytic enzymes were removed from yeast extracts by adsorption on bentonite.

3. The consecutive use of Zn^{2+} ions and bentonite provided a mild method for the isolation of ribonucleic acids and ribonucleoprotein in which the ribonucleic acids had not been enzymically degraded.

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The Biosynthesis of Phospholipids by Human Blood Cells

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For studies on the phospholipids of human blood cells information is required both on the chemical structures of the individual molecules and on their respective metabolic activities. Although the functions of cellular phospholipids are not well defined, these compounds form an integral part of all cell membranes. Finean (1957) has discussed the structure of nerve myelin, in which the ratio of cholesterol:phospholipid:cerebroside is 2:2:1. Lovelock (1955*a*) showed that phospholipid diffuses readily from human blood cells in considerable quantities when these are washed or treated with alumina. Moreover, a constant phospholipid:cholesterol ratio is necessary for the maintenance of the integrity of the cells (Lovelock, 1955*b*). The differences, if any, between the roles of the different classes of phospholipids, e.g. kephalins, lecithins and sphingomyelins, are still obscure.

It has been shown that ^{14}C-labelled acetate is incorporated into rabbit blood cells *in vivo* (Altman, Whatman & Salomon, 1951) and *in vitro* (Altman, 1953). James, Lovelock & Webb (1957) showed that acetate was incorporated into the fatty acids of human blood cells *in vitro*. The incorporation of [^{32}P]orthophosphate *in vivo* into phospholipids of human blood cells has been studied (Tuttle, Scott & Lawrence, 1939; Erf, Tuttle & Lawrence, 1941; Lawrence, Erf & Tuttle, 1941), but there appears to have been little work on the incorporation *in vitro*.

In the following experiments two approaches to lipid metabolism were combined and the concomitant incorporations of [^{32}P]orthophosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells were studied. The separation of human blood cellular phospholipids into the main classes by chromatography on silicic acid is described. Analyses were made of the fatty acids in each class. Moreover, an attempt was made to measure the metabolic activity of each class both from the point of view of the fatty acid chains and the phosphate groups.

METHODS

Incubation of blood. Freshly drawn whole blood was added to a mixture of acid–citrate–dextrose (0·73 M-trisodium citrate, 0·35 M-citric acid, 0·1 M-glucose; 15 ml./100 ml. of whole blood); [^{32}P]orthophosphate (20–50 μc/ml.); sodium [Me-^{14}C]acetate (3–10 μc/ml., specific activity

13·7 μc/mg.); ethylenediaminetetra-acetic acid (1 drop of 0·1% soln./100 ml. of blood); penicillin (100 i.u./ml.) and streptomycin (100 i.u./ml.). Sterile procedures were used throughout. The mixture was incubated at 34·5° with gentle agitation.

Relative rates of phosphate and acetate incorporations. Whole blood was incubated with radioactive phosphate and acetate as described above. The total volume was 140 ml. Samples (10 ml.) were withdrawn at different time intervals (Fig. 1). The cells were separated by centrifuging, washed with 0·9% sodium chloride soln. (3 ml.) and poured slowly into 100 ml. of ethanol–ether (2:1, v/v). After 24 hr. at room temperature the mixture was stored at 2° until required. Solid matter was removed by filtration and the extract concentrated to dryness *in vacuo* in a rotary evaporator. The residue, which was obtained as a thin film on the inside of the flask, was washed with acetone (1 ml.) which was removed after 5 min. This was repeated once. The acetone extracts, which contained only about 2% of the lipid phosphorus, were not investigated further. The residue was washed with ethanol (2 ml.), which was removed after 5 min. This was repeated once and the flask again washed with ethanol (1 ml.). The white insoluble residue suspended in the ethanolic extract was removed by centrifuging. A portion of the ethanolic extract was evaporated to dryness on a weighed aluminium planchet which was then reweighed. The radioactivity of the sample was then estimated.

The counts from ^{14}C indicated in Fig. 1, where the phospholipid was estimated by weighing, were corrected for self-absorption.

Preparation of phospholipids for chromatographic analysis. Whole blood was incubated with radioactive isotopes as described above. The total volume was approx. 100 ml. After 6·5 hr. the cells were separated by centrifuging, washed with 0·9% sodium chloride soln. (20 ml.) and poured slowly into 1 l. of ethanol–ether (2:1, v/v). After 24 hr. at room temperature the solid matter was removed by filtration and concentrated to dryness *in vacuo* in the rotary evaporator. The residue was washed with acetone (10 ml.), which was removed after 5 min. This was repeated once. Ethanol (10 ml.) was added and removed after 5 min. The residue was extracted again in this manner with 10, 20 and 10 ml. of ethanol respectively. The combined ethanolic extracts were centrifuged to remove white suspended residue and then accurately diluted to 50 ml. This extract contained 0·19 m-mole of phosphorus.

Chromatography on silica-impregnated paper. Phospholipids containing 0·19 μmole of phosphorus were chromatographed on silica-impregnated paper with 20% (v/v) of methanol in chloroform as described by Lea, Rhodes & Stoll (1955). The separated components were detected by exposing the paper to iodine vapour. Nitrogenous kephalins were detected by spraying with ninhydrin followed by heating at 95° for 15 min. Choline-containing lipids were detected by development with phosphomolybdic acid followed by reduction with stannous chloride (Chargaff, Levine & Green, 1948).

Radioautography. Kodirex X-ray films were used and the chromatogram was kept in contact with the film for approximately 40 days.

Chromatography on silica-gel column. The method was that described by Hanahan, Dittmer & Warashina (1957), silica gel prepared as described by Lea *et al.* (1955) being used. The column (25 in. × 1 in. diam.) contained 150 g. of silica gel. Phospholipid (0·17 m-mole of phosphorus; approx. 140 mg.), extracted from the cells from approx. 100 ml. of whole blood, was fractionated. Eluate fractions (approx. 8 ml.) from the column were diluted accurately to 10 ml. and 0·1 ml. was transferred to a polythene planchet and evaporated to dryness. The radioactivity of each fraction was estimated (Fig. 2).

Estimation of radioactivity. Samples of combined fractions (Fig. 2) were transferred to polythene planchets, evaporated to dryness and counted with an end-window Geiger tube. The samples were then recounted with the planchets shielded from the Geiger tube by a calibrated foil of phosphorbronze. Previous calibration established that the shield prevented the ^{14}C β particles from being counted and reduced the counts from ^{32}P by a factor of 0·894. From the two measurements and this factor the counts due to ^{14}C and ^{32}P respectively were calculated. The sample was quantitatively washed off the planchet and the phosphorus estimated by the method of Fiske & Subbarow (1925). A calibration curve was constructed with blood phospholipid containing ^{14}C. This indicated that, assuming an average molecular weight of 800, the self-absorption of β particles from ^{14}C was negligible.

Methanolysis of phospholipids. Dry phospholipid dissolved in anhydrous methanolic 7% (w/v) hydrogen chloride was heated at 104° in a sealed tube for 3 hr. After neutralization with excess of Ag_2CO_3 the mixture was filtered. The solid was washed twice with 1 ml. portions of methanol and then twice with 1 ml. portions of water. After addition of water (8 ml.) the combined filtrate and washings were extracted four times with 4 ml. portions of light petroleum (b.p. 40–60°). More light petroleum (4 ml.) was added to the extracts, which were then dried over anhydrous sodium sulphate.

Detection of the bases. The aqueous extract after methanolysis and extraction with petroleum was concentrated to dryness and the residue extracted twice with 0·5 ml. of methanol. The methanolic extract was concentrated to 0·2 ml. and chromatographed on Whatman no. 1 paper with the solvents butanol–acetic acid–water (63:10:27, by vol.) and phenol–water (5:2, v/v) in an atmosphere of ammonia. Bases were detected by treating the chromatogram with ninhydrin or with phosphomolybdic acid and stannous chloride (Chargaff *et al.* 1948).

Analysis of the fatty acid and methyl esters. These were separated and estimated by gas chromatography with a column of Celite coated with ethylene glycol–adipic acid resin (Lipsky & Landowne, 1958; James, 1958). Argon was the carrier gas and the column was fitted with an ionization detector (Lovelock, 1958) and automatic recorder.

RESULTS

Over the course of 10 hr. both phosphate and acetate were incorporated into the phospholipid fraction (Fig. 1). Chromatography of the doubly labelled phospholipids on silica-impregnated paper showed the presence of three components with R_F values and staining properties corresponding to kephalin (R_F 0·92), lecithin (R_F 0·75) and sphingomyelin (R_F 0·55). Radioautography revealed that most of the radioactivity was in the region of the

kephalin. Lecithin was strongly radioactive and there was a very small quantity of radioactivity in a position corresponding to sphingomyelin. A strongly radioactive component remained at the origin. That the radioactivity detected was due to ^{32}P (half-life 14·3 days) and not to ^{14}C (half-life 5030 years) was demonstrated by repeating the radioautography 7 weeks later, when it was found to have disappeared. The radioactivity due to ^{14}C was not detectable under the conditions used.

A larger quantity (approx. 140 mg.) of the doubly labelled phospholipids, obtained after incubation of blood with the radioactive isotopes for 6·5 hr., was fractionated on a column of silica gel, the fractions being eluted with chloroform–methanol mixtures. The radioactivities of the eluate fractions are shown in Fig. 2. The fractions were grouped according to their radioactivities into 12 major fractions as shown. Fraction 12 was obtained by extracting the extruded column at room temperature with methanol. The yields and the relative incorporations of phosphate and acetate into these fractions are given in Table 1.

The fractions were subjected to methanolysis and the bases in each identified by chromatography on paper. Ethanolamine, after treatment with methanolic hydrogen chloride, yielded one component detectable after chromatography and treatment with ninhydrin [R_F 0·20 in butanol–acetic–acid water (solvent *A*) and R_F 0·91–0·92 in phenol–ammonia (solvent *B*)]. This was indistinguishable from ethanolamine and was detected in the products of methanolysis of fractions 3, 4 and 5. Similar treatment of serine yielded two components (R_F 0·10 and 0·20 in *A*, 0·40 and 0·91 in *B*). The first of these, which was the major product, was

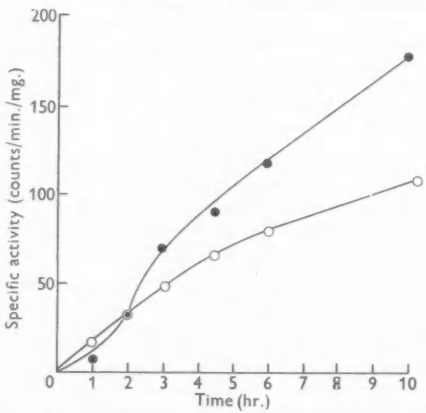

Fig. 1. Incorporation of inorganic [^{32}P]phosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells. ●, Counts due to ^{32}P; ○, counts due to ^{14}C.

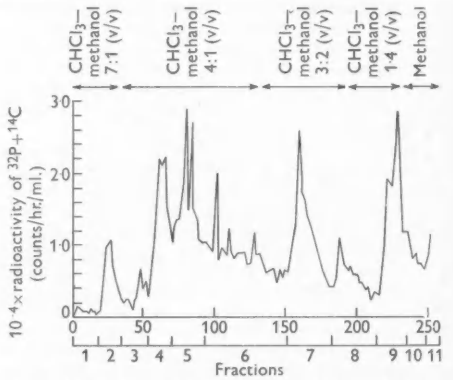

Fig. 2. Chromatography on a column of silica gel of phospholipids from human blood cells. The numbers on the upper abscissa indicate the number of fractions (volume 8 ml. approx.) collected. The numbers on the lower abscissa indicate how these fractions were grouped.

Table 1. *Incorporation of phosphate and acetate into phospholipid fractions of human blood cells*

Fraction	Yield of phosphate (μmoles)	Base	Specific activities (counts/min./μmole of phosphate) ^{32}P	Specific activities (counts/min./μmole of phosphate) ^{14}C	$10^{-3}\times$Total counts (yield × specific activity) ^{32}P	$10^{-3}\times$Total counts (yield × specific activity) ^{14}C	Total counts from ^{32}P / Total counts from ^{14}C
1	1·0	—	0	0	0	0	—
2	1·0	—	5100	466	5·10	0·47	11·0
3	17·4	Ethanolamine	442	81	7·68	1·41	5·5
4	12·5	Ethanolamine	3620	300	45·2	3·75	12·1
5	3·5	Ethanolamine and serine	15700	1220	54·9	4·27	12·9
6	10·0	Serine	8880	800	88·8	8·00	11·1
7	35·0	Choline	980	100	34·2	3·50	9·8
8	20·5	Choline	930	160	19·1	3·28	5·8
9	7·5	Choline, serine and others	2770	330	20·8	2·48	8·4
10	2·0	Choline, serine and others	500	480	1·0	0·96	10·4
11	1·0	Choline and others	560	350	0·56	0·35	1·6
12	4·0	Choline, serine and others	4410	100	17·6	0·40	44·0

Table 2. *Analyses of fatty acids of phospholipid fractions from human blood cells*

Acid	Fractions (see Table 1 and Fig. 2) 3	4	5	6	7	8	9	12
Lauric	—	—	—	1·8	—	—	0·7	1·9
Myristic	—	—	—	—	2·3	1·5	1·4	5·9
Palmitic	2·7	22·6	19·8	11·4	5·2	30·2	29·2	50·9
Unknown	—	—	—	5·4	—	—	—	—
Stearic	18·2	14·7	19·6	17·7	2·2	9·6	8·3	16·2
Oleic	29·2	30·1	22·9	—	5·0	7·9	4·2	15·9
Linoleic	10·5	15·0	6·2	3·1	53·4	7·6	30·6	6·3
Arachidic	—	—	—	—	—	3·1	2·1	—
C_{20} unsaturated (i)	—	—	—	—	—	—	1·6	—
C_{20} unsaturated (ii)	—	—	13·9	—	—	—	—	—
Arachidonic	39·5	17·8	8·3	5·7	22·7	—	—	—
Behenic	—	—	9·4	55·1	—	7·6	4·6	2·2
C_{22} unsaturated	—	—	—	—	—	—	—	—
C_{24} unsaturated (i)	—	—	—	—	—	16·4	5·5	—
C_{24} unsaturated (ii)	—	—	—	—	—	16·2	8·3	—
C_{24} unsaturated (iii)	—	—	—	—	4·9	—	—	—
C_{24} unsaturated (iv)	—	—	—	—	4·3	—	—	—

indistinguishable from serine. The second was indistinguishable from the methyl ester of serine. Serine was detected in the methanolysates of fractions 5, 6, 9, 10 and 12. An unknown ninhydrin-reacting component of R_F 0·13–0·14 (*A*) and 0·57–0·60 (*B*) was detected in fractions 9, 10 and 12. In addition to this, fraction 9 contained two components of R_F 0·33 and 0·47 (*A*) and 0·71 and 0·82 (*B*). The latter component contained choline. Fraction 12 contained a component of R_F 0·17 (*A*) and 0·63 (*B*), which gave a yellow stain when treated with ninhydrin.

The products of methanolysis of fractions 7, 8, 10 and 12 contained choline and chromatography of these indicated the presence of a choline-containing component (R_F 0·24–0·26) distinguishable from choline in *A* (R_F 0·15–0·17) but not in *B* (R_F 0·89–0·90). A choline-containing component which did not contain phosphate but had the same R_F values was obtained from the methanolysis of egg lecithin and the treatment of choline with methanolic hydrogen chloride at 110° for 17 hr. It seems likely, therefore, that this component was methoxycholine. Fraction 11 contained choline and components which gave a purple stain with ninhydrin, but there was insufficient material for chromatographic analysis. Fractions 7 and 8 were distinguished by chromatography on silica-impregnated paper and found to contain choline and sphingomyelin respectively. The distribution of the fatty acids up to the C_{24} acids in each fraction are shown in Table 2.

DISCUSSION

The chromatography on a silica-gel column gave separations into ethanolamine-containing kephalins, serine-containing kephalins, phosphatidylcholine and sphingomyelin. The first two classes came off the column in the reverse order to that found by Hanahan *et al.* (1957) for liver phospholipids. The ratios of the yields, expressed as moles of phosphate, of kephalin, lecithin and sphingomyelin (44:35:21) were close to the molar yields obtained by Hack (1947) from phosphate and choline estimations on unfractionated blood phospholipids.

There were large differences in the distribution of the fatty acids. The most abnormal distribution was shown by the serine-containing lipids, which contained very little unsaturated fatty acids. These serine-containing lipids (fraction 6) had a very high metabolic activity and also a high proportion (55·1%) of an acid with a retention volume corresponding to behenic acid. Fractions 3 and 4, which both contained ethanolamine, were different; fraction 4 had over eight times as much palmitic acid and half as much arachidonic acid as fraction 3.

All the fractions isolated contained both ^{32}P and ^{14}C and therefore contained lipids which had been metabolically active, the activity involving both the phosphate- and carbon-containing parts of the molecules. When phosphate incorporation into each fraction is considered, the kephalins contained 67% of the isolated phospholipid ^{32}P; the lecithins contained 12%, the sphingomyelins 6·5% and fraction 9, 7%. The acetate incorporations were in the same order, 54% being in the kephalins, 11% in the lecithins, 10% in the sphingomyelins and 7·6% in fraction 9. Fraction 9 could be an unknown phospholipid as it contained choline, serine and other substances which stained purple with ninhydrin. It did not haemolyse red cells, and was therefore unlikely to contain appreciable lysophosphatides.

Two different ethanolamine-containing fractions were isolated (fractions 3 and 4) (Table 1). Fraction 4

had 5·9 times the phosphate and 2·7 times the acetate incorporation of fraction 3. Both these fractions developed strong purple colours when treated with Feulgen reagent, indicating the presence of plasmalogens (Feulgen & Bersin, 1939). Collins & Wheeldon (1957) isolated two classes of mixtures of methyl esters of dinitrophenylkephalins from both rat liver and egg yolk. The first class had a phosphorus:dinitrophenol ratio of 1·0 and the second of 2·0. The second class, when derived from rat-liver homogenates incubated with [^{32}P]phosphate, had incorporated more radioactivity than the first class or the corresponding choline-containing lipids. It is possible that the same complexities of kephalin structure occur in human blood cells. In these studies no attempts were made to distinguish between the cellular components of blood, e.g. red cells, white cells and platelets, and it is possible that the different contributions of these may be partly responsible for the metabolic heterogeneity observed.

SUMMARY

1. The simultaneous incorporation *in vitro* of [^{32}P]orthophosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells has been measured.

2. The doubly labelled phospholipids obtained after incubation with the radioactive isotopes for 6·5 hr. were fractionated by chromatography on silica gel, into ethanolamine-containing kephalins, serine-containing kephalins, phosphatidylcholine, sphingomyelin and other phospholipids.

3. All fractions were labelled with both ^{32}P and ^{14}C. Of the isolated phospholipid ^{32}P the kephalins contained the most (67 %), followed by the lecithins (12 %) and the sphingomyelins (6·5 %). The incorporation of ^{14}C followed the same order, the kephalins containing 54 %, the lecithins 11 % and the sphingomyelins 10 % of the isolated lipid ^{14}C.

4. The fatty acids of the fractionated phospholipids were analysed by vapour-phase chromatography and different distributions of fatty acids for each fraction were observed.

5. Two ethanolamine-containing phospholipid fractions were isolated. These differed in the rates of phosphate and acetate incorporation, and in fatty acid composition.

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Studies on Sulphatases

25. THE DETERMINATION OF $BaS^{16}O_3{}^{18}O$ BY INFRARED SPECTROSCOPY*

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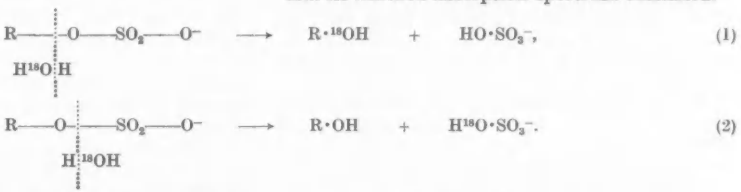
In vitro, the various enzymes of the sulphatase group hydrolyse their specific sulphate ester substrates with the formation of inorganic sulphate and the corresponding desulphated hydroxy compound. Whether this is also their action *in vivo* or whether they can act as transferases is not yet known with certainty. Two transferring activities, involving the transfer of either the sulphate group or the desulphated residue, can be envisaged. A knowledge of the position of cleavage of the sulphate esters by

* Part 24: Lloyd (1959).

the enzymes would be helpful in deciding which of these transfer reactions could occur; splitting between the oxygen and sulphur atoms would indicate the possibility of sulphate transfer whereas C—O bond rupture would indicate transfer of the asulphate.



The position of cleavage can be studied by allowing the sulphatase to act on its substrate in the presence of water containing excess of ^{18}O. Splitting of the C—O bond will result in incorporation of ^{18}O into the hydroxy compound formed (1), whereas in O—S bond-splitting the ^{18}O will be found in the liberated sulphate (2). The sulphate can be precipitated as barium sulphate and its ^{18}O content assessed.

In a previous study with arylsulphatases the ^{18}O content of barium sulphate was measured by combustion with carbon and mass-spectrographic determination of the ^{18}O content of the carbon dioxide formed (Spencer, 1958). Extension of this study to other sulphatases was hampered by the lack of ready access to a mass spectrometer and alternative methods of determining the ^{18}O content of barium sulphate were therefore considered. Infrared-absorption spectrometry seemed promising since the substitution of the heavier ^{18}O atom for ^{16}O in barium sulphate should lead to a decrease in the frequencies of vibration in the molecule with consequent shifting of the infrared absorption peaks to longer wavelengths. The magnitude of the shift can be calculated from the Teller–Redlich equation. The free sulphate ion is a tetrahedral XY_4 molecule belonging to the point group T_D and for the fully isotopically substituted $XY_4{}^{(i)}$

$$\frac{\omega_1}{\omega_1} = \frac{\omega_2^{(i)}}{\omega_2} = \sqrt{\frac{m_Y^{(i)}}{m_Y}} \qquad (3)$$

for the totally symmetrical (ν_1) and doubly degenerate (ν_2) vibrations where ω_1 and ω_2 are the frequencies of the ν_1 and ν_2 vibrations respectively, m_Y is the mass of the Y atom and all the quantities marked with the superscript (i) refer to the isotopic material (Herzberg, 1945, p. 233). For $S^{16}O_4$ and $S^{18}O_4$ $\omega_1^{(i)}/\omega_1 = 0{\cdot}942$ and since ν_1 for the free $S^{16}O_4$ ion is 981 cm.$^{-1}$ (Table 1) the expected shift of this vibration in $S^{18}O_4$ will be 57 cm.$^{-1}$ As a rough approximation the shift of ν_1 for $S^{16}O_3{}^{18}O$ will be one-quarter of this value, i.e. 14 cm.$^{-1}$, and a shift of this magnitude in the region around 1000 cm.$^{-1}$ should be readily discernible with most infrared spectrophotometers. To check whether these predicted shifts might be of practical use for the detection of $BaS^{16}O_3{}^{18}O$, a sample of barium sulphate containing 87 % of $BaS^{16}O_3{}^{18}O$ was prepared and its infrared-absorption spectrum examined.

EXPERIMENTAL

Materials. Potassium *p*-nitrophenyl sulphate was prepared by the method of Burkhardt & Lapworth (1926). Water containing 10 and 90 atoms % excess of ^{18}O was obtained from The Radiochemical Centre, Amersham, Bucks and the Weizmann Institute of Science, Rehovoth, Israel.

Preparation of $BaS^{16}O_3{}^{18}O$. Potassium *p*-nitrophenyl sulphate (52 mg.) was dissolved in 54·8 μl. of water containing 90 atoms % excess of ^{18}O and acidified with 2·8 μl. of 10 N-HCl. The arylsulphate was completely hydrolysed by heating the solution, which was contained in a 3 mm. int. diam. tube in a boiling-water bath for 15 min. The non-immersed part of the tube was cooled in a stream of air to prevent excess of evaporation. After cooling, the hydrolysed material was diluted with 1 ml. of water, extracted with 1 ml. of ether ($\times 4$) to remove *p*-nitrophenol and then warmed to expel dissolved ether. The liberated sulphate was precipitated by the addition of 1 ml. of 5 % (w/v) $BaCl_2$ and the barium sulphate formed was washed on the centrifuge with 2 ml. of N-HCl ($\times 2$), 2 ml. of water ($\times 5$), 2 ml. of ethanol ($\times 2$) and finally dried *in vacuo* over P_2O_5 for 2 days. It has previously been established that 100 % splitting of the O–S bond occurs in the acid hydrolysis of *p*-nitrophenyl sulphate (Spencer, 1958) and, taking into account the water added in the 10 N-HCl, the barium sulphate isolated in the present experiment was calculated to contain 21·75 atoms % excess of ^{18}O or 87 % of $BaS^{16}O_3{}^{18}O$. The $BaS^{16}O_3{}^{17}O$ content was 1·2 % and the $BaS^{16}O_4$ content, by difference, was 11·8 %. The majority of the work was carried out on this specimen, but some confirmatory spectra were obtained with a sample containing 90 % of $BaS^{16}O_3{}^{18}O$. This was prepared by passing sulphur trioxide into water containing 90 atoms % excess of ^{18}O and precipitating and washing the barium sulphate as before.

Samples (20 mg.) containing different amounts of $BaS^{16}O_3{}^{18}O$ were prepared by mixing the 87 % isotopic material with normal barium sulphate. To obtain homogeneous mixtures on this small scale the components were weighed into a small centrifuge tube, suspended in 2 ml. of ethanol and the tube was vibrated against a flattened nail rotating at about 1500 rev./min. After centrifuging, the ethanol was removed and the mixture dried *in vacuo* over P_2O_5.

Infrared-absorption spectrophotometry. Spectra were studied on a double-beam Perkin–Elmer Infracord with a rock-salt prism. Accurate measurements of the frequencies

of the absorption peaks were obtained on a double-beam Perkin-Elmer Model 21 with a rock-salt prism for frequencies above 700 cm.$^{-1}$ and a single-beam Perkin–Elmer model 12C with a KBr prism for frequencies below 700 cm.$^{-1}$ Liquid paraffin (Nujol) mulls were used throughout.

Arylsulphatase of Aspergillus nidulans. *Aspergillus nidulans* (C.M.I. 16643) was grown on Czapek–Dox synthetic medium for 6 days with aeration. The mycelium was harvested, washed with water and starved by aeration in water for 5 hr. The mycelium was then ground in a mortar with acetone at 0° and allowed to stand in acetone at this temperature for 3 hr. The mycelium was separated by filtration, washed well with cold acetone and dried *in vacuo*. The supernatant of an aqueous suspension (2·5%, w/v) of the acetone-dried material was dialysed against several changes of water at 2° and then freeze-dried. The lyophilized material hydrolysed 3·91 μmoles of *p*-nitrophenyl sulphate/hr./mg. at 38° in 0·1 M-acetate buffer, pH 6·2, with 0·015 M-substrate.

Arylsulphatase assay. A final volume of 20 μl. of 0·5 M-acetate buffer, pH 6·2, containing potassium *p*-nitrophenyl sulphate (0·015 M), the enzyme and the inhibitor where appropriate, was incubated at 38° for 1 hr. The reaction was stopped by the addition of 100 μl. of 0·5 M-NaOH and, after centrifuging, the absorption at 400 mμ was measured in microcells with the Hilger Uvispek spectrophotometer. The amount of *p*-nitrophenol liberated was calculated by the use of ϵ_{400} 18 200.

RESULTS

Method developed for the determination of $BaS^{16}O_3{}^{18}O$

The frequencies of the infrared absorption peaks of normal barium sulphate and barium sulphate containing 87% of $BaS^{16}O_3{}^{18}O$ are shown in Table 1. The significance of the spectra are discussed later but, of practical interest, it was noted that whereas the 981 and 1076 cm.$^{-1}$ frequencies of $BaS^{16}O_4$ were shifted to 961 and 1059 cm.$^{-1}$ in $BaS^{16}O_3{}^{18}O$, a weak peak at 981 cm.$^{-1}$ and a shoulder at 1072 cm.$^{-1}$ were also shown in the isotopic sample (Table 1 and Fig. 1). Since the isotopic sample contained 11·8% of $BaS^{16}O_4$ in addition to the $BaS^{16}O_3{}^{18}O$ it seemed reasonable to attribute the subsidiary peak and the shoulder to the presence of the normal barium sulphate.

This detection of 11·8% of $BaS^{16}O_4$ in the presence of 87% of $BaS^{16}O_3{}^{18}O$ suggested that observations at 981, 961, 1076 and 1059 cm.$^{-1}$ might be used to estimate the amounts of normal and isotopic barium sulphate in a given sample. The peaks at 1076 and 1059 cm.$^{-1}$, however, proved of little use for this purpose since even the best resolution, obtained with the sample containing 11·8% of $BaS^{16}O_4$ and 87% of $BaS^{16}O_3{}^{18}O$ with the Perkin–Elmer 12C spectrophotometer, succeeded only in showing the presence of the minor component as a slight shoulder on the major peak. In a sample containing 60% of $BaS^{16}O_4$ and 40% of $BaS^{16}O_3{}^{18}O$ the specific peaks at 1076 and 1059 cm.$^{-1}$ respectively were indistinguishable and a new flat peak between the two appeared (Fig. 1). On the other hand, the ν_1 peak in both $BaS^{16}O_4$ and $BaS^{16}O_3{}^{18}O$ was sharp and in various mixtures of normal and isotopic barium sulphate peaks at 981 and 961 cm.$^{-1}$ were clearly resolved (Fig. 1).

Table 1. *Infrared spectra of normal barium sulphate and barium sulphate containing* 87% *of* $BaS^{16}O_3{}^{18}O$

Assignments for barium sulphate are from Ramdas (1954). The fundamental frequencies of the free sulphate ion are known from Raman spectra in solution (Kohlrausch, 1943).

Barium sulphate			Free $S^{16}O_4{}^{2-}$ ion		
Frequencies (cm.$^{-1}$)					
$BaS^{16}O_4$	$BaS^{16}O_3{}^{18}O$ sample	Assignment	Assignment	Degree of degeneracy	Frequencies (cm.$^{-1}$)
981	961	ω_1^1 (B_{1u})	ν_1 (a_1)	1	981
?	?	ω_1^2 (B_{3u})			
	(981 weak)				
?	?	ω_2^2 (B_{2u} or B_{1u})	ν_2 (e)	2	451
?	?	ω_2^3 (B_{1u} or B_{2u})			
?	?	ω_2^4 (B_{3u})			
1076	1059	ω_3^1 (B_{1u})	ν_3 (f_2)	3	1104
1112	1095	ω_3^2 (B_{3u})			
1187	1189	ω_3^3 (B_{1u})			
?	?	ω_3^4 (B_{3u})			
?	?	ω_3^5 (B_{2u})			
	(1072 shoulder)				
611	604	ω_4^1 (B_{1u})	ν_4 (f_2)	3	613
637	634	ω_4^2 (B_{1u})			
?	?	ω_4^3 (B_{1u})			
?	?	ω_4^4 (B_{3u})			
?	?	ω_4^5 (B_{2u})			

A method for the determination of $BaS^{16}O_3{}^{18}O$ in barium sulphate was developed by utilizing the resolution of the ν_1 frequency. The principle is illustrated in Fig. 2, which shows part of the spectrum of a paraffin oil (Nujol) mull of barium sulphate containing 8·7 % of $BaS^{16}O_3{}^{18}O$. The values of *A* and *B* (Fig. 2) from the log I°/I scale were taken to represent the absorption due to normal and isotopic barium sulphates respectively. The percentage of $BaS^{16}O_3{}^{18}O$ in the sample is given by $100\ B/A+B$ and in the case illustrated the measurements gave 9 %. Although based on the Lambert–Beer law this method can only be approximate since it also depends to some extent upon the freehand completion of the $BaS^{16}O_3{}^{18}O$ and $BaS^{16}O_4$ spectra as shown by the dotted lines in Fig. 2. To make the method more sensitive concentrated mulls requiring about 20 mg. of sample have been used and a 0·05 mm. spacer has been placed between the rock-salt plates. In this way the extinction of the ν_1 peaks is effectively increased but at the same time the base of the peaks is broadened and the freehand completion of the spectra becomes more approximate. In practice a compromise is made by limiting the extinction of the strongest ν_1 peak to 0·5. A linear magnification of *A* and *B* (Fig. 2) can be obtained by adjusting the spectrophotometer so that the trace at the approximate position *C* has an extinction of about 0·05.

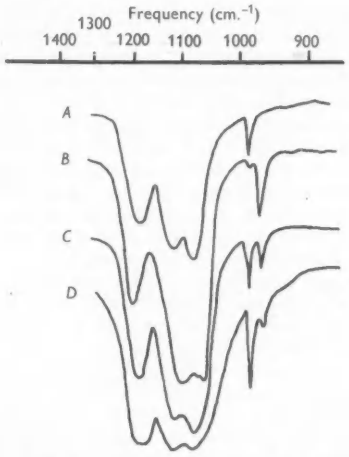


Fig. 1. Infrared-absorption spectra of Nujol mulls of barium sulphate samples. *A*, Normal barium sulphate; *B*, barium sulphate containing 87 % of $BaS^{16}O_3{}^{18}O$ and 11·8 % of $BaS^{16}O_4$; *C*, 40 % of $BaS^{16}O_3{}^{18}O$, 59·5 % of $BaS^{16}O_4$; *D*, 8·7 % of $BaS^{16}O_3{}^{18}O$, 91·2 % of $BaS^{16}O_4$.

A straight line was obtained when the known $BaS^{16}O_3{}^{18}O$ content of prepared samples was plotted against the percentage of the isotopic material calculated from the spectra (Fig. 3). The presence of as little as 2 % of $BaS^{16}O_3{}^{18}O$ (i.e. 0·5 atom % excess of ^{18}O) could be detected and an accuracy varying between 2 atoms % of $BaS^{16}O_3{}^{18}O$

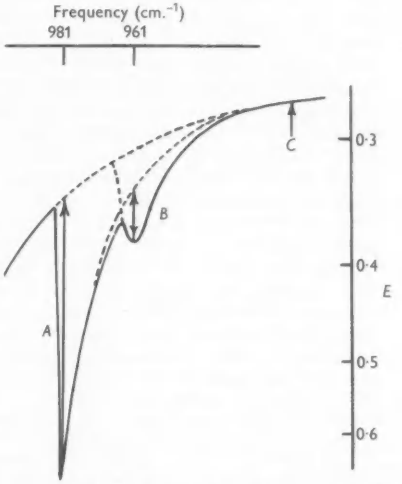


Fig. 2. A portion of the spectrum of a Nujol mull of a barium sulphate sample containing 8·7 % of $BaS^{16}O_3{}^{18}O$. The mull was prepared with 20 mg. of sample and a 0·05 mm. spacer was placed between the rock-salt plates of the cell. For details see text.

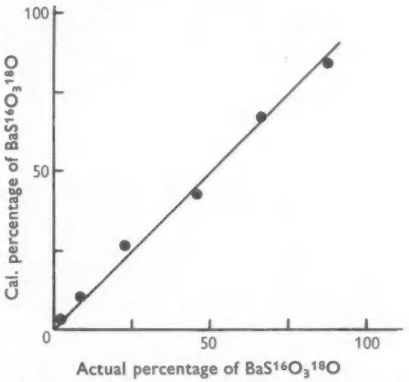


Fig. 3. Spectra of prepared samples of barium sulphate containing various amounts of $BaS^{16}O_3{}^{18}O$ were examined; 20 mg. samples and a 0·05 mm. spacer were used. The percentages of $BaS^{16}O_3{}^{18}O$ calculated from the spectra are plotted against the known concentration.

for low values of isotopic barium sulphate and 4 atoms % of $BaS^{16}O_3{}^{18}O$ for high values can be considered a reasonable claim.

Biological application of the method

Mechanism of action of the arylsulphatase of Aspergillus nidulans. To check the practical efficiency of the infrared method of $BaS^{16}O_3{}^{18}O$ estimation the position of cleavage of potassium *p*-nitrophenyl sulphate by the arylsulphatase of *A. nidulans* was studied. The reaction mixture contained 12 mg. of lyophilized *A. nidulans* preparation, 30 mg. of potassium *p*-nitrophenyl sulphate and 0·136 g. of sodium acetate trihydrate in 2 ml. of ^{18}O-enriched water and the whole was adjusted to pH 6·2 with a measured amount of acetic acid by using a glass electrode. The final concentration of ^{18}O in the water of the reaction mixture was calculated to be 8·7 atoms % excess. After incubating at 38° for 3 hr. the liberated inorganic sulphate was precipitated as barium sulphate and washed free of protein, *p*-nitrophenol and unhydrolysed substrate as detailed previously (Spencer, 1958). The barium sulphate (yield 18·2 mg.) was analysed by the infrared spectroscopic method and was found to contain 7·8 % of $BaS^{16}O_3{}^{18}O$. The theoretical value for O–S cleavage of *p*-nitrophenyl sulphate was 8·7 % of $BaS^{16}O_3{}^{18}O$.

Effect of ions on the arylsulphatase of Aspergillus nidulans. Arylsulphatases have been divided into two types on the basis of their relative substrate specificities and their behaviour towards certain ions (Dodgson & Spencer, 1957). Type I enzymes are scarcely affected by phosphate, sulphate or fluoride but are markedly inhibited by cyanide whereas the type II enzymes behave in the opposite manner. The effect of these and other ions on the arylsulphatase activity of *A. nidulans* (Table 2) shows that this enzyme can be classified as a type I arylsulphatase.

Table 2. *Inhibition of the arylsulphatase activity of* Aspergillus nidulans

Activity was measured against 0·015 M-potassium *p*-nitrophenyl sulphate in 0·5 M-acetate buffer, pH 6·2, over 1 hr. at 38°. The inhibitors (0·02 M final concentration) were adjusted to the assay pH by means of a glass electrode before being added to the incubation mixture.

Compound added	Inhibition (%)
KCN	65·5
KH_2PO_4	1·3
K_2SO_4	1·2
NaF	19·9
Na_2SO_3	48·7

DISCUSSION

The conventional crystallographic unit cell of barium sulphate contains four molecules of barium sulphate and this is also the primitive or Bravais cell (Bhagavantam & Venkatarayudu, 1951) from which selection rules may be deduced. The free sulphate ion, which belongs to the tetrahedral point group T_D, has nine fundamentals which give rise to four distinct frequencies, a_1 (single), e (doubly degenerate) and f_2 (two, both triply degenerate). In the barium sulphate crystal the symmetry of a single sulphate molecule is changed to the monoclinic local symmetry C_s and the result is a perturbation of the internal vibrations of the sulphate ion thereby removing the degeneracy and giving rise to nine separate frequencies. The number of internal modes of the four sulphate ions in the primitive barium sulphate cell will therefore be 36. Since there is a centre of symmetry the mutual exclusion rule applies and it can be shown that of the expected frequencies 15 will be infrared active, 18 Raman active and 3 inactive. Thus in the infrared spectrum of barium sulphate two bands near 981 cm.$^{-1}$, three bands near 451 cm.$^{-1}$, five bands near 1104 cm.$^{-1}$ and five bands near 613 cm.$^{-1}$ can be expected. Of these predicted bands only six were actually observed (Table 1). None of the weakly infrared-active components of ν_2 was seen either in the normal or isotopic barium sulphate. Duval & Lecomte (1954) with barium sulphate and Hass & Sutherland (1956) with gypsum also failed to observe the ν_2 bands, but they are evident in the sulphates of copper, magnesium and manganese (Duval & Lecomte, 1954). Ramdas (1954), using barytes crystals in the 001 and 110 cleavage sections, observed nine of the 15 expected infrared fundamentals including two components of ν_2, and a further band is known from reflexion spectra. In the present study slight differences in the frequencies of the overtone and combination bands of the normal and isotopic barium sulphates were observed in the region 2000–3000 cm.$^{-1}$ but an accurate analysis was not attempted.

Absorption spectra of mulls do not allow a more specific assignment of the observed frequencies than that given in Table 1, and an exact comparison of the observed shifts with the theoretical shifts calculated from the Teller–Redlich equation is not possible. However, it is generally considered (Herzberg, 1945, p. 238) that for both small and large mass differences no crossing over of frequencies of the same species can occur because of the repulsion in consequence of Fermi resonance, and it is therefore permissible to correlate the absorption frequencies of the normal and isotopic barium sulphates as shown. The band frequencies observed for the isotopic material may not

necessarily be the actual fundamental frequencies of $BaS^{16}O_3{}^{18}O$ but may be intermediate peaks between those of $BaS^{16}O_3{}^{18}O$ (87 %) and the $BaS^{16}O_4$ (11·8 %) present in the sample. This is more likely for the strongly absorbing broad ν_3 peaks but, on the other hand, the ν_1 peaks of $BaS^{16}O_4$ and $BaS^{16}O_3{}^{18}O$ are clearly resolved.

The infrared spectrophotometric method for the determination of $BaS^{16}O_3{}^{18}O$ is insensitive in comparison with the mass-spectrographic method (Hoerring, 1952; Spencer, 1958), which can readily distinguish differences of 0·01 atom % excess of ^{18}O. Clearly, the infrared method can be of little use with samples containing low concentrations of ^{18}O or when small differences in isotope content between two samples are required to be measured, but for the study of the position of cleavage of sulphate esters by sulphatases, where an 'all or none' response can be expected, the method offers the advantages of speed and simplicity. For experiments of this type it is advisable to carry out the enzyme reactions in water containing at least 5 atoms % excess of ^{18}O, and the availability of such material constitutes the major limiting factor in the use of the method.

Application of the method to the study of the hydrolysis of *p*-nitrophenyl sulphate by the arylsulphatase of *Aspergillus nidulans* showed that the enzyme acts by cleaving the O–S bond of the arylsulphate. On theoretical grounds (cf. Spencer, 1958) it may therefore be deduced that if the enzyme can act as a transferase it will transfer the sulphate rather than the phenol group. Inhibition studies indicate that the enzyme, which is similar to the arylsulphatase of Taka-diastase (*Aspergillus oryzae*), is a type I arylsulphatase and its method of action is also the same as other type I enzymes, namely rat-liver arylsulphatase C and the arylsulphatase of *Alcaligenes metalcaligenes*, which are already known to split the O–S bond. No actual transferring activity has yet been demonstrated for any type I arylsulphatase.

SUMMARY

1. The infrared-absorption spectra of normal barium sulphate and barium sulphate containing 87 % of $BaS^{16}O_3{}^{18}O$ have been examined.

2. A method of estimating $BaS^{16}O_3{}^{18}O$ in barium sulphate has been developed, based on the measurement of relative extinctions at 981 and 961 $cm.^{-1}$

3. The method has been used to analyse the barium sulphate prepared from the inorganic sulphate liberated by *Aspergillus nidulans* arylsulphatase acting on potassium *p*-nitrophenyl sulphate in the presence of water containing 8·7 atoms % excess of ^{18}O. The $BaS^{16}O_3{}^{18}O$ content of the barium sulphate was found to be 7·8 %, showing that the type I arylsulphatase of *Aspergillus nidulans* splits the O–S bond of arylsulphates.

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The Acyl-Group Specificity of Choline Acetylase

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Although much is known about the specificity of the cholinesterases, that of choline acetylase, the enzyme responsible for the synthesis of acetylcholine, has received relatively little attention. The alkyl-group specificity of purified rat-brain choline acetylase has been investigated by Burgen, Burke & Desbarats-Schonbaum (1956) with a number of choline analogues; limited studies of the acyl-group specificity of the enzyme in pigeon brain have been made by Gardiner & Whittaker (1954) and Whittaker (1953) and in invertebrates by

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Nachmansohn & co-workers (Korey, de Braganza & Nachmansohn, 1951; Berman, Wilson & Nachmansohn, 1953) and Frontali (1958).

It is now well established that the synthesis of acetylcholine from acetate and choline by nervous tissue *in vitro* occurs in two steps: first, the activation of acetate by acetic thiokinase in the presence of adenosine triphosphate and coenzyme A to form acetyl coenzyme A; secondly, the transfer of the acetyl group from coenzyme A to choline by choline acetylase. [The nomenclature recommended in *Biochem. J.* (1956) **64**, 782, is used here.] In the present investigation, the acyl-group specificity of sheep-, pigeon- and rat-brain choline acetylase preparations was studied by coupling the enzyme to a crude acyl thiokinase preparation from pigeon liver, capable of activating all the fatty acids investigated. This necessitated preliminary experiments designed to establish the activation spectrum of the liver preparation and the concentration of adenosine triphosphate, coenzyme A, inorganic ions and acyl thiokinase needed to ensure that formation of acyl-coenzyme A was not rate-limiting. The acyl thiokinase activity of the crude brain preparations was also studied; the rate of activation of fatty acids was found, with some species variation, to decrease markedly with increasing chain length, thus emphasizing the need for supplementation with the liver thiokinase.

The use of an acyl-coenzyme A donor system was preferred to the use of stoicheiometric amounts of synthetic acyl-coenzyme A derivatives because there is good evidence that the latter do not give, even in purified systems, as high a rate of acylcholine synthesis as is obtainable with the donor system. Thus Whittaker (1953) found that the rate of acetylcholine synthesis from acetyl-coenzyme A by crude pigeon-brain preparations was less than 10% of that obtained in the presence of pigeon-brain acetic thiokinase, and Berman *et al.* (1953) reported that purified squid-ganglion choline acetylase synthesized acetylcholine from acetyl-coenzyme A at only 30% of the rate obtained with a donor system consisting of acetylphosphate, coenzyme A and bacterial phosphotransacetylase. Similar observations have been made by Smallman (1958) on rabbit brain. The reason for this effect is not fully understood, but may be related to the presence of acetate acceptors other than choline.

The results presented here show that crude brain preparations were able to synthesize all the *n*-acylcholines up to C_6 (the highest tested) and also palmitoylcholine. The products of the reaction were identified chromatographically. Liver preparations alone were unable to synthesize the lower choline esters, but could form palmitoylcholine at a higher rate than brain. Pigeon brain was selected for detailed study because of its high activity, and fractionation by the procedure of Kumagai & Ebashi (1954) revealed the presence of three enzymes. One, for which the term 'choline acetylase' is reserved, catalysed the formation of acetyl- and propionyl-choline only, the latter at about 30% of the acetylcholine rate, and corresponded to the enzyme in guinea-pig brain purified by Kumagai & Ebashi. The enzyme responsible for the synthesis of the intermediate homologues (up to C_6) was inactivated by the purification procedure, and its acyl-group optimum was not determined. It is proposed that this enzyme be provisionally termed 'choline acylase II'. The third enzyme, recovered in a discarded fraction from the Kumagai & Ebashi purification, was responsible for the synthesis of palmitoylcholine. Its acyl-group optimum was not determined and the provisional designation 'choline acylase III' is proposed. Since choline acetylase and choline acylase II are analogous to acetocholinesterase and pseudocholinesterase respectively, it was of interest to see whether a palmitoylcholinesterase analogous to choline acylase III could be identified in tissues. This proved to be so, and the distribution of the enzyme in different tissues was studied.

Of interest in view of the frequent use of cysteine as a component of the reaction mixture in choline acetylase determinations was the finding that *NS*-diacetylcysteine was formed from acetyl-coenzyme A and cysteine in the presence of liver extracts. The formation of this compound may give rise to discrepancies between the ferric hydroxamate (Hestrin 1949) and bioassay methods of measuring choline-ester formation. Competition between cysteine and choline for acyl-coenzyme A derivatives was avoided by replacing cysteine by borohydride.

METHODS

Preparation of acetone-dried powders

Acetone-dried powders of pigeon and sheep livers (intended primarily for activation studies) were prepared essentially as described by Kaplan & Lipmann (1948). Acetone-dried powders of pigeon and sheep brain, sheep caudate nucleus and rat tissues were prepared by pounding the tissue in a cold mortar with 10 vol. of acetone at $-8°$ to $-15°$ followed by homogenization in a high-speed blendor for 2–5 min. (cf. Hebb, 1955).

Activation studies

For activation studies, acetone-dried powders were extracted with 0·02 M-$KHCO_3$ (20–100 mg./ml.), and the supernatant fluid, separated by centrifuging at 1200 *g* for 3–5 min. at 0°, was used as the source of enzyme. The rate of activation was measured with hydroxylamine as a trapping agent for activated acyl groups or spectrophotometrically as described below. The reaction mixture (I) contained in 2·0 ml., unless otherwise stated, $MgCl_2$ (20 μmoles), KCl (100 μmoles), coenzyme A (CoA) (Pabst,

70% pure) (0·1 μmole), adenosine triphosphate (ATP) (Pabst, dipotassium salt, free of non-adenine nucleotides) (10 μmoles), KBH_4 (1 μmole), fatty acid (20 μmoles) as ammonium (palmitate) or sodium salt (other acids), hydroxylamine hydrochloride (500 μmoles). The reagents were adjusted to pH 7·0 with 2N-NaOH before adding enzyme. The pH was selected with subsequent choline-acetylase experiments in view. The mixture was incubated for 1 hr. at 37°. The acylhydroxamic acid formed was estimated by the procedure of Beinert *et al.* (1953). The reaction mixture was cooled to 0°, 1 ml. of 3N-HCl and 1 ml. of $FeCl_3$ [5% (w/v) $FeCl_3,6H_2O$ in 0·1N-HCl] were added, the suspension was centrifuged and the extinction at 540 mμ compared with those of zero-time samples and tubes from which ATP and CoA had been omitted. Activation of palmitate was measured by the method of Lipmann & Tuttle (1950) or in some experiments by that of Lipmann & Tuttle (1945*a*) by using larger quantities of CoA and adding hydroxylamine at the end of the incubation. The amount of activation occurring was measured by the increase in acylhydroxamate concentration during the incubation, resulting from the presence of CoA and ATP (corrected for acylhydroxamate formation from endogenous fatty acid).

Choline-acylase studies

Extraction and incubation. The extraction of the enzyme from brain acetone-dried powders and the composition of the incubation mixture were as described for acyl thiokinase, with the following modifications. The extraction medium was usually 0·155M-NaCl, but in a few preliminary experiments 0·025M-cysteine hydrochloride neutralized with NaOH in 0·19M-NaCl or, neutralized with KOH, in 0·19M-KCl, or 0·0028M-KH_2PO_4–0·0042M-K_2HPO_4 buffer (pH 7·0) in 0·05M-KCl (Nachmansohn & John, 1945) was used as indicated. The reaction mixture (II) contained, in addition to $MgCl_2$, KCl, CoA, ATP, KBH_4 and fatty acid in the same concentrations as in (I), eserine sulphate (0·1 μmole), $KHCO_3$ extract from 20 mg. of acetone-dried pigeon liver (0·2 ml.) and extract of acetone-dried brain. Choline chloride (20 μmoles) replaced hydroxylamine as acyl-group acceptor. Controls containing no brain extract showed that pigeon liver alone was unable to acylate choline except in the presence of palmitate.

Assay. The ester formed during the incubation was estimated in 1 ml. samples of the reaction mixture by the method of Hestrin (1949) (short-chain esters) or Lipmann & Tuttle (1950) (palmitate). Short-chain esters were also estimated after deproteinization by bioassay with the rectus abdominis muscle of the frog. Samples were chilled, brought to pH 4–4·5, heated at 100° for 10 min., cooled and centrifuged and the supernatant fluid was assayed by the method of Chang & Gaddum (1933) as modified by Feldberg & Hebb (1947), with acetylcholine as standard. The activity divided by the ester content of a sample (in molar units) gave the relative molar potency of the ester which could be compared with that of an authentic sample of the expected ester, synthesized as described by Keyl, Michaelson & Whittaker (1957), as a means of identifying the product of the enzyme reaction. Choline (free and esterified) was estimated by treating deproteinized samples with an equal volume of saturated aqueous ammonium reineckate at 0°, collecting the precipitate by centrifuging, dissolving it in 2 ml. of acetone and measuring $E_{540\ m\mu}$.

Chromatographic identification of choline esters. This was carried out by the methods of Gardiner & Whittaker (1954) and Whittaker & Wijesundera (1952). For column chromatography, a deproteinized sample of the reaction mixture was placed on a column of Amberlite XE-97 buffered to pH 4·3, 0·6 cm. in diameter and 10–12 cm. in height, and eluted with 0·1M-NaH_2PO_4. Esters were located in successive fractions of the eluate by bioassay. Portions of eluted material were freeze-dried and extracted three times with 3 ml. of cold ethanol, the extracts evaporated and taken up in the least volume of mM-acetate buffer, pH 4·5. The solution was passed through De-Acidite FF (The Permutit Co. Ltd., London, −16+50 mesh) in the chloride form to replace other anions by chloride, applied to Whatman no. 1 filter paper and chromatographed in butanol saturated with water by the ascending method. Palmitoylcholine and palmitic acid were applied in ethanol and run similarly. Compounds were located by exposure to iodine vapour, by spraying with hydroxylamine and $FeCl_3$ (Whittaker & Wijesundera, 1952), or bromocresol purple (Reid & Lederer, 1951), or by elution of successive areas and assay on the frog rectus.

Purification of choline acylases

Pigeon-brain choline acetylase. This was purified as described by Kumagai & Ebashi (1954).

Pigeon-liver choline acylase III. This was purified from $KHCO_3$ extracts of liver acetone powders by $(NH_4)_2SO_4$ fractionation. Precipitates were dissolved in the minimum volume of 0·02M-$KHCO_3$ and dialysed against 0·02M-$KHCO_3$. All operations were carried out at 0°.

Protein. This was estimated by the method of Kalckar (1947) or by a micro-Kjeldahl procedure.

Spectrophotometric studies

Fatty acid activation and acylation of choline were followed spectrophotometrically by measuring the change in $E_{230\ m\mu}$ due to the formation and disappearance of the thio ester group of acyl-CoA. Measurements were made in the Hilger Uvispek spectrophotometer; the reaction was followed discontinuously, as described by Mahler, Wakil & Bock (1953) and Hele (1954), or continuously, as described by Stadtman (1953), and satisfied the conditions regarding the final thiol–thio ester ratio discussed by Kielley, Stadtman & Bradley (1954). When followed discontinuously, the reaction mixture was as already described for choline acetylase (mixture II) except that the initial volume was 3·0 ml., KCl was omitted, 0·9 μmole of CoA was used, the concentration of pigeon-liver thiokinase was adjusted to give approximately equal rates of activation with different substrates, lower (C_2–C_6) fatty acids were added as their potassium salts and choline and eserine were added after a steady-state concentration of acyl-CoA had been attained. Samples (0·2 ml.) were withdrawn at zero time and successive 5 min. periods thereafter, added to 0·8 ml. of 0·1M-KH_2PO_4–K_2HPO_4 buffer (pH 7·2), or immersed in a boiling-water bath for a few minutes, diluted with 3·0 ml. of water and filtered. When followed continuously, the reaction mixture consisted of $MgCl_2$ (3 μmoles), CoA (0·1 μmole), ATP (3 μmoles), KBH_4 (1 μmole), ammonium salt of the fatty acid (6 μmoles) and enzyme in a total volume of 3·0 ml. When the activation reaction was complete, choline chloride (2 μmoles) and eserine sulphate

(0·1 μmole) were added and the decline in extinction accompanying the transfer of the acyl group from acyl-CoA to choline was observed.

Esterase studies

Esterase measurements were carried out at 37°, in 0·023 M-$NaHCO_3$ buffered to pH 7·4 with CO_2, by the Warburg manometric technique as modified by Ammon (1933). Gum acacia (0·2%) was added as stabilizer in experiments with purified enzymes. Activity was expressed in Q units (i.e. μl. of acid produced/mg. dry wt. of enzyme/hr.) or as μl./100 mg. wet wt. of tissue/hr. Harvard plasma fraction IV-6 ($Q_{Acetylcholine}$ 690), kindly supplied by Dr D. M. Surgenor, or Cholase brand purified human-plasma cholinesterase (Cutter Laboratories) ($Q_{Acetylcholine}$ 3300) and purified bovine-red-cell cholinesterase (Winthrop Stearns Inc.) ($Q_{Acetylcholine}$ 97 000) served as sources of purified butyro- and aceto-cholinesterase respectively. For tissue distribution studies, 10% (w/v) homogenates of pigeon and rat tissues were prepared in 0·023 M-$NaHCO_3$ and preserved at 0° in the presence of a few drops of chloroform until required. With palmitoylcholine as substrate activity declined rapidly after 10 min. even in the presence of gum acacia. Accordingly, activity measurements were made only during the initial 10 min. period with this substrate.

RESULTS

Activation studies

Effect of sodium ions on activation. Sodium ions (Korff, 1953) have been stated to be inhibitory to acetic thiokinase; yet NaCl has been used by previous workers to extract choline acetylase from brain tissue. Accordingly, the effect of this ion on acetate activation by pigeon liver was studied. Table 1 shows that NaCl had no inhibitory action on acetate activation and it was therefore thought legitimate to use NaCl for extracting choline acetylase. In experiments with brain, no difference was found in the rate of activation or choline ester synthesis whichever of the extractants mentioned in the Methods section was used. Brain and liver acetic thiokinase activities were additive.

Activation of various fatty acids by liver and brain. Table 2 shows the relative abilities of extracts of acetone-dried powders of pigeon and sheep liver and brain and rat liver to activate a series of fatty acids. The results show that in acetone-dried powders of brain from both pigeon and sheep activation declines rapidly with increasing chain length, though sheep brain appears to be more active on the longer-chain fatty acids than is pigeon brain. On the other hand, in acetone-dried powders of liver of all three species, activation tends to increase with increasing chain length. In the light of recent studies by Vignais, Gallagher & Zabin (1958), acetone-dried powders may not represent the best preparation for studying fatty acid activation by brain for all substrates. The main purpose of these experiments was to find out what contribution, if any, to the total activating potential

Table 1. *Effect of sodium and potassium ions on acetate activation by extracts of pigeon-liver acetone-dried powder*

Reaction mixture I as described in the text was used with the following modifications: sodium acetate was replaced by the potassium salt; hydroxylamine hydrochloride was neutralized with KOH; volume of extract 0·2 ml., equivalent to 20 mg. of powder and containing 2·0 mg. of N; NaCl (200 μmoles) was added and KCl omitted as indicated. Acetate activation was measured by hydroxamate formation.

	Rate of acetate activation (μmoles/g. of powder/hr.)
Complete system without additions	42·5
NaCl added	43·0
NaCl added, no KCl	45·0

Table 2. *Activation of various fatty acids by extracts from acetone-dried powders of pigeon and sheep liver and brain and rat liver*

Reaction mixture I as described in text was used. Volumes of extracts: pigeon liver, 0·2 ml., equivalent to 20 mg. of powder, containing 2·0 mg. of N; pigeon brain, 1·0 ml., equivalent to 20 mg. of powder, 1·9 mg. of N; sheep liver, 0·5 ml., equivalent to 20 mg. of powder, 0·69 mg. of N; sheep brain, 0·5 ml., equivalent to 10 mg. of powder, 1·77 mg. of N; rat liver, 0·5 ml., equivalent to 10 mg. of powder.

	Rate of acylhydroxamate formation (μmoles/g. of powder/hr.)				
	Pigeon		Sheep		Rat
	Liver	Brain	Liver	Brain*	Liver
Acetate	51·0	16·5	28·0	18·1	25·0
Propionate	37·5	7·0	34·5	9·4	23·0
n-Butyrate	40·5	2·5	36·0	9·4	26·0
n-Valerate	54·0	2·0	82·0	5·4	38·0
Hexanoate	69·0	0·5	125·0	5·4	47·0
Palmitate	80·0	0	62·0	5·4	69·0

* Similar values were obtained with sheep caudate nucleus.

Table 3. *Properties of reaction product, compared with* NS-*diacetylcysteine*

Observations: +, positive; −, negative; a, not investigated.

	Reaction product	Same after alkaline hydrolysis	Synthetic *NS*-diacetyl-cysteine	Same after alkaline hydrolysis	Cysteine
R_F in butanol saturated with water	0·11	0	0·12	0	0
Fe-hydroxamate reaction in alkaline solution	+	−	+	−	−
Ninhydrin reaction	−	+	−	+	+
Nitroprusside reaction	−	+	−	+	+
Frog-rectus assay	−	−	−	−	−
Solubility	Ether+	a	Ether+	a	a
Absorption maximum (mμ)	231	a	231	a	a
$10^{-3}\,\varepsilon_{231\,m\mu}$	4·06	a	4·10 3·90*	a	a

* Noda *et al.* (1953).

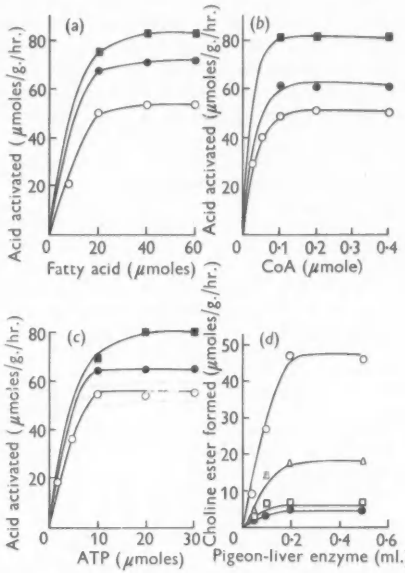

Fig. 1. Dependence of activating enzyme of pigeon liver on (*a*) substrate, (*b*) CoA and (*c*) ATP, and of crude pigeon-brain choline-acetylase preparation on (*d*) pigeon-liver activating enzyme. ○, Acetate; △, *n*-butyrate; □, *n*-valerate; ●, hexanoate; ■, palmitate. Reaction mixtures: (*a*, *b*, *c*) as in Table 2, except that concentrations of fatty acid, CoA and ATP were varied as indicated; (*d*) reaction mixture II, using 1·0 ml. of extract of pigeon brain (equivalent to 20 mg. of acetone-dried powder) except that the volume of pigeon-liver extract was varied as indicated.

of the coupled system the brain extracts would make. They emphasize the desirability of coupling a liver-activating system with brain for the study of the acyl specificity of the brain choline acylase.

Since the pigeon-liver preparation was the most active per mg. of acetone-dried powder, it was used as the source of activating enzyme, and the optimum conditions for its use for acylcholine synthesis were studied as described in the next section.

Investigation of optimum conditions for synthesis of acylcholine. In order to obtain conditions in which the activation reaction was not rate-limiting in choline-ester synthesis, the dependence of the activation reaction on concentrations of fatty acid, CoA and ATP was determined. The results are shown in Fig. 1*a–c*. The rate of activation by the pigeon-liver system was not much changed after the amounts of CoA exceeded 0·1 μmole, ATP, 10 μmoles and fatty acid, 20 μmoles. These amounts were therefore used. Also (Fig. 1*d*) choline-ester formation was not increased with these concentrations of substrate and cofactors by raising the volume of pigeon-liver preparation above 0·2 ml., equivalent to 20 mg. acetone-dried powder. Choline-ester formation was also unchanged by increases in CoA, ATP and fatty acid concentrations above those required for optimum activation.

Formation of NS-*diacetylcysteine.* In initial experiments when 20 μmoles of neutralized cysteine hydrochloride were used in each tube in place of borohydride as a sulphydryl-group reductant, there was a discrepancy between the acetylcholine assays by the frog rectus and the Hestrin (1949) methods. In the absence of either choline or an extract of brain acetone-dried powder, no acetylcholine was formed as measured by the bioassay procedure, yet the Hestrin (1949) procedure showed the formation of 20 μmoles of ester/g. of powder/hr. The formation of the compound was CoA- and ATP-dependent and ceased if cysteine was replaced by potassium borohydride. The properties of the compound are summarized in Table 3.

The R_F in butanol saturated with water was close to that of acetylcholine. The alkaline hydrolysis product was identified as cysteine. Accordingly, *NS*-diacetylcysteine was prepared by the method of Neuberger (1938). Both the reaction product and the synthetic compound had the absorption maximum at 231 mμ characteristic of these esters and the molar extinction coefficients of both specimens were close to that reported by Noda, Kuby & Lardy (1953) for *NS*-diacetylcysteine. The mechanism of formation of the compound was assumed to be as follows: acetyl-CoA reacts with cysteine either non-enzymically (Stadtman, 1952; Stadtman & White, 1953) or via pigeon-liver thioltransacetylase (Brady & Stadtman, 1954) to form *S*-acetylcysteine. This compound is unstable and becomes rearranged to *N*-acetylcysteine, which could then offer the sulphydryl group once more as an acetyl-group acceptor to form the stable *NS*-diacetylcysteine. Since its formation is CoA-dependent, it is unlikely that thiolesterase (Strecker, Mela & Waelsch, 1955) is involved.

Acyl-group specificity of crude and purified brain-choline-acetylase preparations

Specificity of crude brain preparations. Table 4 (columns 2, 4, 5 and 6) shows the ability of crude brain-choline-acetylase preparations from pigeon, sheep and rat to synthesize choline esters from a series of fatty acids in the presence of pigeon-liver acyl thiokinase and optimum concentrations of the components of the activating system. It will be seen that all these preparations are able to synthesize all the choline esters up to *n*-valerylcholine though at decreasing rates relative to acetylcholine. Pigeon and sheep brain could also synthesize hexanoylcholine. Pigeon and rat brain were also able to synthesize palmitoylcholine. The rates for acetylcholine synthesis are within the ranges reported by Hebb (1957). Unlike the other esters palmitoylcholine was also synthesized by the liver preparation alone; in Table 4 the contribution of the liver enzyme has been subtracted from the total palmitoylcholine synthesis.

Table 4. *Specificity of crude and purified brain-choline-acetylase preparations*

Reaction mixture II as described in the text was used. Vol. of brain extracts: unpurified extract from whole pigeon brain, 1·0 ml., equivalent to 20 mg. of powder, containing 1·9 mg. of N; 22-fold purified pigeon-brain choline acetylase, 0·1 ml., equivalent to 26 mg. of powder, containing 0·06 mg. of N and 0·22 mg. of protein; extract of sheep brain, 0·5 ml., equivalent to 10 mg. of powder, 0·88 mg. of N; sheep caudate nucleus, 0·3 ml., equivalent to 5·5 mg. of powder, 0·10 mg. of N; rat brain, 0·5 ml., equivalent to 50 mg. of powder.

	Choline-acylase activity (μmoles/g. of powder/hr.)				
	Pigeon		Sheep		Rat
	Whole brain unpurified	Purified choline acetylase	Whole brain	Caudate nucleus	Whole brain
Acetate	47·0	26·0	35·0	89·0	21·6
Propionate	15·0	8·3	28·0	87·2	20·2
n-Butyrate	11·0	0	25·0	37·4	17·0
n-Valerate	7·0	0	21·0	33·8	3·2
Hexanoate	5·0	0	14·0	20·3	0
Palmitate	6·4*	0	0	0	9·6*

* Corrected for contribution to total palmitoylcholine synthesis by pigeon-liver extract.

Table 5. *Comparison of properties of choline esters synthesized by pigeon-brain extract with those of synthetic esters*

	Synthetic choline ester					Isolated choline ester			
		Retention vol. (ml.)					Retention vol. (ml.)		
Compound	R_F	Elution peak	Displacement peak	Relative molar potency	Fatty acid added	R_F	Elution peak	Displacement peak	Relative molar potency
Choline	0·08	25	—	—	None	0·08	20	—	—
Acetylcholine	0·10	40	—	100	Acetate	0·12	36	—	100
Propionylcholine	0·22	55	—	154	Propionate	0·25	55	—	150
n-Butyrylcholine	0·28	60	—	125	*n*-Butyrate	0·29	60	—	123
n-Valerylcholine	0·31	140	—	40	*n*-Valerate	0·30	130	—	33
Hexanoylcholine	0·36	>160	2	20	*n*-Hexanoate	0·34	—	7	15

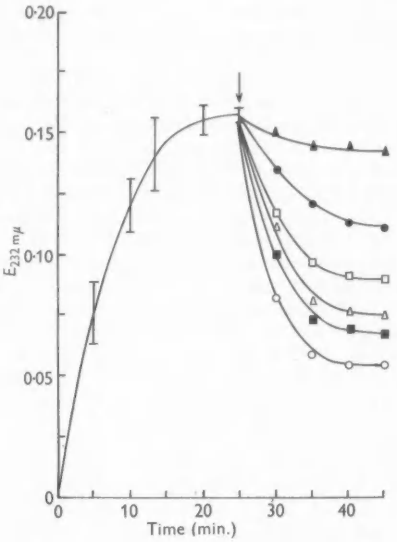

Fig. 2. Spectrophotometric determination of fatty acid activation by pigeon-liver and -brain extracts and deacylation in the presence of choline. The reaction medium was as described in the text for discontinuous spectrophotometry and contained 0·2–0·6 ml. of extract of pigeon-liver acetone-dried powder, equivalent to 20–60 mg. of powder and containing 2·0–6·0 mg. of N, and 1·0 ml. of extract of pigeon brain, equivalent to 20 mg. of powder, 1·9 mg. of N. Choline and eserine were added at ↓. ○, Acetate; △, propionate; □, *n*-butyrate; ●, *n*-valerate; ▲, hexanoate; ■, palmitate. The concentration of liver extract was adjusted to give approximately equal rates of activation; the points on this part of the curve are averages irrespective of substrate, and the bars through the points represent the range of values averaged.

Characterization of products. Since fatty acids can in certain circumstances be oxidized to acetyl-CoA, it was important to identify the products of the reaction, although it was observed that no reduction of di- or tri-phosphopyridine nucleotide occurred in the presence of fatty acids with any of the preparations studied. Accordingly, these were characterized by chromatographic and, where possible, biological tests. Some results are shown in Table 5. The R_F values, retention volumes and relative molar potencies of the esters synthesized by the pigeon-brain preparation are all in good agreement with those of the corresponding synthetic esters, which in turn are close to the values obtained by Keyl *et al.* (1957). Palmitoylcholine, not included in Table 5, proved to be difficult to identify chromatographically owing to its ability to form stable complexes with palmitic acid. Its identification is discussed in a section below describing its synthesis by liver preparations.

Spectrophotometric studies. The activation and acylation reactions were further studied in a stepwise manner as shown in Fig. 2, which shows the increase in $E_{232\,m\mu}$ due to the formation of acyl-CoA and the subsequent decrease in extinction due to the disappearance of acyl-CoA as the acyl group is transferred to choline. For all fatty acids, a steady-state concentration of acyl-CoA appears to be attained by 20 min. Presumably the steady-state concentration of acyl-CoA represents a balance between continued synthesis and destruction by deacylases and other enzymes present in the preparation. On adding choline, the steady-state concentration is rapidly lowered to a new level, and the difference in the two steady-state concentrations may be taken as the amount of choline ester synthesized. That this is correct is shown by Table 6, which shows good agreement between the spectrophotometric results and the amount of ester

Table 6. *Comparison of spectrophotometric, bioassay and chemical methods of determining choline-acylase activity*

Experimental conditions were as in Fig. 2.

Fatty acid	Steady-state acyl-CoA concn. (determined spectrophotometrically) (μmoles/g. of brain)	Choline ester formed (μmoles/g. of brain powder)		
		Spectrophotometric	Bioassay	Colorimetric
Acetate	58·0	37·0	41·0	45·0
Propionate	58·0	32·0	32·0	30·0
n-Butyrate	56·0	24·0	24·0	22·0
n-Valerate	60·0	20·0	18·0	14·0
Hexanoate	60·0	9·6	10·0	10·0
Palmitate	57·0	31·0*	—	33·0*

* μmoles/g. of brain + liver powders.

synthesized in the same samples as determined by the bioassay and colorimetric procedures, with the appropriate synthetic esters as standards. The results for palmitate here represent the combined effect of brain and liver.

Specificity of purified preparations. In order to investigate whether the spectrum of activity in Table 4 was due to a single enzyme or a mixture of enzymes, acetone-dried powder extracts of brain were purified by the method of Kumagai & Ebashi (1954). Pigeon brain was selected for this study because of its high activity/mg. of acetone-dried powder. The purified enzyme (column 3) proved to be able to synthesize only acetyl- and propionyl-choline. The rate of synthesis of acetylcholine by the purified preparation (0·57 mg. of acetylcholine chloride/mg. of protein/hr.) is comparable with that reported by Kumagai & Ebashi. The discarded fractions were therefore examined for choline-acylase activity. None of the fractions showed activity when coupled with a pigeon-liver system containing butyrate, valerate or hexanoate. However, palmitoylcholine synthesis was obtained with the precipitate from the first acetic acid precipitation. These results demonstrate that the specificity pattern shown by the crude pigeon-brain extract is a composite due to at least three separate choline acylases. The first acylase synthesizes acetyl- and to a lesser extent propionyl-choline. It would be appropriate to reserve the term choline acetylase for this enzyme. The second enzyme (choline acylase II) synthesizes butyryl-, valeryl- and hexanoyl-choline. It probably contributes little to the synthesis of propionylcholine, since the ratio of the rates of synthesis of propionyl- to acetyl-choline hardly changed as a result of purification. Whether it makes a contribution to acetylcholine synthesis cannot be determined as yet. This enzyme was either destroyed during the purification or was rendered inactive in some other way, e.g. by removal of an unsuspected cofactor. Palmitoylcholine synthesis seems to be brought about by yet another enzyme (choline acylase III). This enzyme does not catalyse the synthesis of any of the lower acylcholines either in brain or liver, since liver preparations alone, although much more active in synthesizing palmitoylcholine than brain, were unable to synthesize any of the lower acylcholines.

In view of the higher choline acylase III activity of liver, the enzyme was further studied in this tissue.

Pigeon-liver choline acylase III

Cofactor requirements. Table 7 shows the results of attempts to demonstrate the cofactor requirements of choline acylase III. The system requires CoA, ATP and palmitate (columns 4 and 5, rows 2–4). A requirement for choline could not be demonstrated in the crude liver preparation,

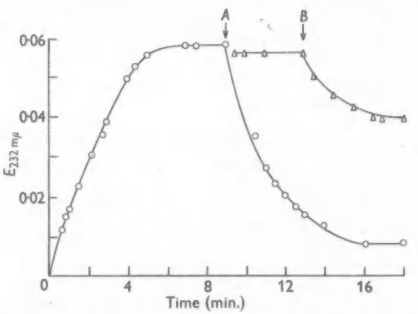

Fig. 3. Palmitoyl-group activation and transfer by a purified pigeon-liver preparation. The reaction mixture was as described in the text for continuous spectrophotometry, and contained 0·5 ml. of purified liver enzyme equivalent to 32·5 mg. of original acetone-dried powder and containing 0·11 mg. of N and 1·13 mg. of protein. At *A*, choline chloride and eserine were added to a portion of the intact system (○) and to a portion boiled at that time (△). Fresh enzyme was added to the boiled system at *B*.

Table 7. *Cofactor requirements for palmitate esterification by crude and purified pigeon-liver preparations*

The complete system was reaction mixture II as described in the text but containing no brain extract, and either 0·2 ml. of extract of pigeon-liver acetone-dried powder (equivalent to 20 mg. of powder) (crude system) or 0·2 ml. of fourfold-purified fraction (equivalent to 20 mg. of original powder and containing 3·16 mg. of protein and 0·39 mg. of N) (purified system).

Conditions	Choline added (μmoles)	Choline recovered from crude system (μmoles)	Ester formed (μmoles/g. of powder)	
			Crude system	Purified system
Complete system	20	23·4	30·1	23·0
Complete system less palmitate	20	25·4	0	0
Complete system less CoA	20	—	0	0
Complete system less ATP	20	—	0	0
Complete system less choline	0	4·3	40·0	0
Complete system less enzyme	20	14·0	0	0

apparently owing to the presence of sufficient quantities of choline in the tissue extract. Column 3 gives the amount of choline recovered as the reineckate from the reaction mixture: 3·4–5·4 (average, 4·4) μmoles of choline were recovered in excess of any which could be accounted for by added choline. This corresponds to 30 mg. of choline chloride/g. of liver powder. The total amount present is probably more, since only 70% of added choline was recovered from the reaction mixture in the absence of enzyme.

An attempt was made to remove choline and to separate the activating enzyme from the choline acylase by fractionating the liver extract with ammonium sulphate. Acylase activity was found in the fractions precipitated when the concentration of ammonium sulphate was raised from 30 to 50 g./100 ml. of extract, but these fractions also contained the thiokinase activity. Refractionation increased the specific activity without separating the enzymes. However, the choline dependence of the reaction could now be demonstrated, as shown in Table 7, column 5, row 5, and Fig. 3. In the latter, the activation reaction may be seen as the increase in $E_{232\ m\mu}$ and the synthesis of palmitoylcholine as the decrease in extinction on addition of choline chloride and eserine. If the preparation is boiled after a steady-state concentration of acyl-CoA is reached, addition of choline chloride does not cause a fall in extinction until fresh enzyme is added. These results indicate the enzymic and stepwise nature of the reaction.

Chromatographic identification of enzymically synthesized palmitoylcholine. Experiments with mixtures of synthetic palmitoylcholine and palmitic acid showed that these compounds had a strong tendency to form a stable complex. Methods which appeared promising when evaluated with the two compounds separately (e.g. reversed-phase column chromatography, paper electrophoresis) failed to separate mixtures, especially those in which the acid component was in excess, as in the enzyme studies. Thus, although the two compounds had different R_F values in butanol and water (palmitoylcholine 0·70, palmitic acid 1·0), when chromatographed together they tended to travel as a single spot of intermediate R_F. Palmitic acid was tenaciously retained by columns of the strongly basic resin De-Acidite FF (as the chloride form in ethanol), whereas palmitoylcholine passed through. However, only 70% of palmitic acid was retained when palmitoylcholine was also present.

However, in two experiments, enzymically synthesized palmitoylcholine was successfully identified after chromatography as a spot of R_F 0·72 which gave a brown colour with iodine vapour, formed a hydroxamate under alkaline conditions and gave an alkaline reaction with bromocresol purple, reactions given by synthetic palmitoylcholine but not by palmitic acid. The spot was negative to ninhydrin and showed no phosphorus by the method of Hanes & Isherwood (1949); therefore it could not have contained a phospholipid.

The material was isolated from the protein-free supernatant, after incubation, by precipitation with an equal volume of ice-cold saturated aqueous ammonium reineckate. The washed precipitate was taken up in 50% (v/v) aqueous acetone and passed through a column of De-Acidite FF in the chloride form to remove reineckate ion and unesterified palmitate. The effluent material was evaporated to dryness *in vacuo* and taken up in ethanol. Palmitoylcholine was tenaciously retained by Amberlite XE-97 and was extensively hydrolysed on alumina, so that chromatography on these materials was impracticable.

Palmitoylcholine esterase

The existence in brain and liver of a distinct choline acylase concerned with the synthesis of palmitoylcholine raised the question whether this compound was hydrolysed by tissues, and if so, by known cholinesterases or a separate enzyme.

Effect of butyro- and aceto-cholinesterases. Palmitoylcholine was not significantly hydrolysed ($< 0{\cdot}2$% of the acetylcholine rate) by either of the preparations of purified human-plasma butyrocholinesterase. The ester was tested at 2 and 10 mM concentrations by using concentrations of enzyme up to 100 times that required to give a rapid carbon dioxide evolution with 30 mM-acetylcholine. The purified bovine-red-cell acetocholinesterase failed to hydrolyse 10 mM-palmitoylcholine detectably at a concentration 10 times that giving a rapid hydrolysis of 10 mM-acetylcholine (74 μl./10 min.).

Owing to the surface-active nature of palmitoylcholine, it was thought possible that its failure to be hydrolysed might be due to a non-specific inactivation of the enzyme by the substrate, analogous to that observed by Mounter (1952) with cobra-venom cholinesterase and water-immiscible substrates, even though 0·2% of gum acacia had been added as a stabilizing agent. 2 mM-Palmitoylcholine inhibited acetylcholine hydrolysis by cholase by 100%; under similar conditions 30 mM gave 20% inhibition. However, this inhibition was 83% reversible by 1:100 dilution and 67% reversible by dialysis for 15–20 hr.; the smaller inhibition by 30 mM-choline was 100% reversible in parallel experiments. Thus the inhibition of butyrocholinesterase by palmitoylcholine is mainly reversible and the failure of the enzyme to hydrolyse this substrate cannot be ascribed either to enzyme inactivation or to a failure of the enzyme and ester to interact.

Hydrolysis by pigeon and rat tissues. Hydrolysis of palmitoylcholine could be demonstrated manometrically in pigeon and rat tissues with 10 % (w/v) homogenates in aq. 0·2 % (w/v) sodium bicarbonate, as shown in Table 8. The general trend of activity is the same in both species, liver being the most active tissue of those examined, followed by spleen, intestine, kidney and pancreas. Brain, heart, lung and skeletal muscle showed low or negligible activity. There was no correlation between the palmitoylcholinesterase activity of the various tissues and their cholinesterase contents as measured by three different standard substrates. As pigeon liver was the most active source of the enzyme, some additional studies were made with this tissue. The system obeyed the simple Michaelis–Menten kinetics, 10 mM-palmitoylcholine giving a nearly maximum initial velocity. The Michaelis constant was estimated to be 4·0 mM. The enzyme was not inhibited by 10^{-4} M-eserine or 10^{-5} M-di*iso*propyl phosphofluoridate. The insensitivity of the enzyme to these high concentrations of anti-cholinesterases, its different distribution in the tissues from known cholinesterases and the inability of purified aceto- and butyro-cholinesterases to hydrolyse palmitoylcholine is conclusive evidence that the palmitoylcholine-hydrolysing esterase is distinct from other cholinesterases, but its relation to tissue lipases and aliesterases has not been established.

The low level of the enzyme in pigeon brain is contrary to the findings of Gomori (1948). The low level of activity might be due to absence of activating ions such as Ca^{2+}, Mg^{2+} and Co^{2+}, or to destruction of enzyme due to the detergent quality of the substrate or competitive inhibition by excess of substrate, but these explanations were made unlikely by the following observations. Addition of Ca^{2+}, Mg^{2+} and Co^{2+} up to 30 mM failed to increase the esterase activity; no increase of activity occurred even with a substrate concentration as low as 0·5 mM, and ammonium palmitate, also surface-active, produced only 27 % inhibition of pigeon-brain acetocholinesterase at 10 mM.

DISCUSSION

Our results confirm and extend those of Gardiner & Whittaker (1954) and Whittaker (1953) in showing that crude vertebrate-brain choline-acetylase preparations can synthesize a series of homologous choline esters. Unlike the earlier workers, instead of relying on the endogenous acyl thiokinase of brain preparations, we used a fortified system in which acyl-CoA derivatives were supplied at a non-limiting rate by a pigeon-liver acyl thiokinase preparation. In these circumstances, the relative rates of choline-ester synthesis may be taken to provide a true picture of the acyl-group specificity of the enzymes concerned. The work with purified extracts shows that pigeon-brain choline acetylase has a high degree of specificity, being able to synthesize only acetyl- and propionyl-choline, and suggests that the synthesis of the short-chain homologues of these esters is catalysed by a second choline acylase with an apparent acyl-group optimum near butyrate. If choline acetylase be regarded as the counterpart of acetocholinesterase, this enzyme may be that of brain pseudocholinesterase, though it is not known how specific it is for the acyl-group acceptor. The eserine insensitivity and CoA- and ATP-dependence of the reaction would seem to exclude the possibility that this enzyme is pseudocholinesterase acting in reverse (Hestrin, 1950), but its identity with a non-specific thiolesterase which can utilize choline in place of water as an acyl-group acceptor is not excluded. A comparison of the work of Korey *et al.* (1951) with unpurified and those of Berman *et al.* (1953) with purified squid-ganglion preparations suggests that this tissue also contains choline acylase II, though the pharmacologically active material synthesized by the crude ganglion preparation in the presence of butyrate was not

Table 8. *Palmitoylcholine esterase activity of pigeon and rat tissue*

Activities are expressed as μl./100 mg. wet wt. of tissue/hr.

	Pigeon				Rat	
	10 mM-Palmitoyl-choline	30 mM-Butyryl-choline	30 mM-Benzoyl-choline	30 mM-Acetyl-β-methylcholine	10 mM-Palmitoyl-choline	30 mM-Butyryl-choline
Liver	204	670	36	56	164	200
Spleen	—	—	—	—	38	216
Intestine	108	340	200	34	12	1800
Kidney	108	234	0	52	16	162
Pancreas	32	240	76	26	—	—
Brain	6	52	12	2000	4	162
Heart	0	280	0	96	12	840
Lung	0	0	24	8	6	124
Muscle	0	30	0	0	0	6

positively identified by Korey *et al.* as butyrylcholine. The same omission renders dubious the significance of the observation of Torda & Wolff (1945) that a homologous series of fatty acids increased acetylcholine synthesis by minced frog brain. On the other hand, fly brain does not appear to contain the second enzyme (Frontali, 1958).

The physiological significance of the synthesis of pharmacologically active homologues of acetylcholine by brain tissue is not at present clear. Although the results presented here were obtained with fortified systems, there is little doubt that the acyl thiokinase activity of the brain itself is adequate for synthesis of these esters. However, although propionylcholine has been found in ox spleen (Banister, Whittaker & Wijesundera, 1953; Gardiner & Whittaker, 1954; Augustinsson, 1955; Henschler, 1957), it is doubtful whether homologues of acetylcholine occur in brain tissue. Holtz & Schümann (1954) have reported the presence of butyrylcholine in ox brain, but Henschler (1956) has stated that this compound appears only in autolysing brain. M. J. Keyl & V. P. Whittaker (unpublished work) were unable to identify any active acetylcholine homologues in ox, sheep, goat or rabbit brain. Thus if these homologues are capable of being synthesized in brain tissue *in vivo*, there must be an effective mechanism for preventing their accumulating even in small amounts.

The finding of palmitoylcholine-synthesizing and -hydrolysing enzymes distinct from those involved in the metabolism of the lower esters is interesting in relation to Kennedy's (1956) isolation from tissues of an alkali-labile lipid-like compound containing no phosphorus with properties similar to those of palmitoylcholine. According to Kennedy, incorporation of labelled choline into this compound also required ATP and CoA. As with the short-chain homologues of acetylcholine, the significance of palmitoylcholine in brain metabolism is difficult to evaluate. Although the rate of palmitate activation by pigeon brain (the species in which palmitoylcholine synthesis was mainly studied) was too low to be measured, there seems little doubt that brain tissue can activate the longer-chain fatty acids, and optimum conditions may not have prevailed in these experiments. For example, hydroxylamine is known to be inhibitory to activating and other enzymes in certain circumstances (Mahler *et al.* 1953; Hele, 1954; Lipmann & Tuttle, 1945*b*; Raw, 1953). The recent work of Vignais *et al.* (1958) provides additional evidence for palmitate activation in brain.

SUMMARY

1. The acyl-group specificity of crude and purified brain-choline-acetylase preparations from pigeon, sheep and rat has been studied. The preparations were coupled to a pigeon-liver activating system as a source of acyl-coenzyme A derivatives.

2. In preliminary experiments, the activation spectrum of extracts of liver and brain and the optimum conditions for activation were studied in order to ensure that the formation of acyl-coenzyme A derivatives was not a rate-limiting step. The rate of activation by liver extracts increased with increasing chain length, but the reverse was true of brain.

3. Cysteine, used by previous workers as a component of the choline-acetylase system, was found to act as an acyl-group acceptor and was replaced by borohydride.

4. Crude brain preparations were able to synthesize all the choline esters from acetate to hexanoate and also palmitoylcholine. Purification studies showed that three enzymes were involved.

5. Palmitoylcholine synthesis was studied in brain and in liver. It required coenzyme A and adenosine triphosphate as well as palmitate and choline, and the two-stage nature of the synthesis was demonstrated.

6. Palmitoylcholine hydrolysis was catalysed by an enzyme in tissues which was distinct from known cholinesterases.

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Controlled Biosynthesis of Actinomycin with Sarcosine

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The actinomycins represent a family of chromopeptide antibiotics which differ solely in the nature of the amino acids present in the peptides of the molecule (Fig. 1). It has been established that an actinomycin-producing organism generally synthesizes a mixture of these substances. For example, *Streptomyces antibioticus* forms a mixture consisting of actinomycins I–V; occasionally trace amounts of a sixth component are produced (Katz & Goss, 1958). *Streptomyces chrysomallus* produces actinomycins IV, VI and VII (Schmidt-Kastner, 1956*b*).

The quantitative and qualitative nature of the actinomycin mixture synthesized can be modified to a considerable extent; in particular, the nitrogen source supplied has been shown to have a profound influence on its composition. Actinomycin IV increased from 10 to 83 % of the mixture produced by *S. chrysomallus* when DL-valine was added to the medium (Schmidt-Kastner, 1956*b*); hydroxy-L-proline brought about an increase in synthesis of actinomycin I from 6–7 to 31 % of the actinomycins produced by *S. antibioticus* (Katz & Goss, 1958). New actinomycins were formed by *S. chrysomallus* when DL-isoleucine or sarcosine was added to the medium (Schmidt-Kastner, 1956*a*). In our Laboratory it has been determined that *S. antiobiticus* forms at least one new actinomycin with sarcosine and several new compounds when DL-pipecolic acid is used.

During an investigation of the role of amino acids on actinomycin synthesis by *S. antibioticus*, it was observed that sarcosine selectively stimulated the production of actinomycins II and III (Fig. 2). These components, normally synthesized in trace amounts, represented approximately 60 % of the actinomycin mixture formed under the nutritional conditions employed. By paper-chromatography techniques, it was established that actinomycin II contains threonine, valine, sarcosine, and *N*-methylvaline, whereas actinomycin III possesses, in addition, one-half the amount of proline found in actinomycin IV (Katz, Goss & Pugh, 1958). Recently Johnson & Mauger (1959) obtained quantitative data showing that 4 moles of sarcosine and no proline are present in actinomycin II, and

that 3 moles of sarcosine and only 1 mole of proline are in actinomycin III. They proposed for actinomycins II and III the structures indicated in the legend of Fig. 1.

Schmidt-Kastner (1956*b*) has suggested that exogenous sarcosine interfered with the incorporation of proline into certain actinomycin peptides. The results obtained in our studies provide evidence for the view that sarcosine competes with proline and replaces it in certain actinomycin peptides.

EXPERIMENTAL

Culture. Streptomyces antibioticus, strain 3720, was used throughout the investigation. The procedure for preparation of an inoculum has been described previously (Goss & Katz, 1957). An N-Z amine medium consisting of N-Z amine A (a pancreatic digest of casein prepared by Sheffield Chemical Co., Norwich, N.Y., U.S.A.), 25 g., Bacto beef extract 10 g., tap water 1 l., pH 7·0, was employed for growth of the organism.

Production medium. A chemically defined medium containing L-glutamic acid 2·0 g., D-galactose 10·0 g., K_2HPO_4 1·0 g., $MgSO_4,7H_2O$ 0·025 g., $CaCl_2,2H_2O$ 0·025 g., $ZnSO_4$, $7H_2O$ 0·025 g., $FeSO_4,7H_2O$ 0·025 g., distilled water 1 l., pH 7·2–7·3, was used for actinomycin production. Amounts (100 ml.) of medium were distributed into 250 ml. Erlenmeyer flasks and sterilized at 15 lb./in.^{2} pressure and 121° for 15 min. D-Galactose was autoclaved separately in a similar manner and added to the medium just before inoculation. Production of actinomycin was carried out in shaken cultures (240 rev./min.) at 28°. Aqueous solutions of sarcosine and other amino acids generally were added at the onset of actinomycin synthesis (1–3 days after inoculation), unless specified otherwise.

Determination of actinomycin potency. The antibiotic titre of culture filtrates was determined by a paper-disk method of bioassay (Goss & Katz, 1957).

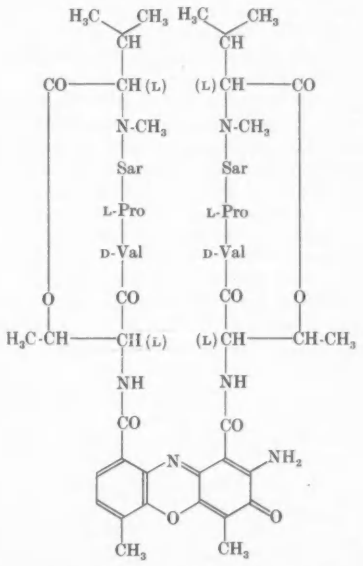

Fig. 1. Actinomycin IV. Sequence of amino acids: L-threonine, D-valine (D-Val), L-proline (L-Pro), sarcosine (Sar), *N*-methyl-L-valine (Brockmann *et al.* 1956; Bullock & Johnson, 1957). Actinomycin I. 1 mole of proline is replaced by 1 mole of hydroxyproline (Brockmann & Pampus, 1955). Actinomycin II. 2 moles of proline are replaced by 2 moles of sarcosine (Johnson & Mauger, 1959). Actinomycin III. 1 mole of proline is replaced by 1 mole of sarcosine (Johnson & Mauger, 1959). Actinomycin V. 1 mole of proline is replaced by 1 mole of 4-oxoproline (Brockmann & Manegold, 1958). Actinomycin VI. 1 mole of D-valine is replaced by 1 mole of D-*allo*isoleucine (Brockmann *et al.* 1956). Actinomycin VII. 2 moles of D-valine are replaced by 2 moles of D-*allo*isoleucine (Brockmann *et al.* 1956).

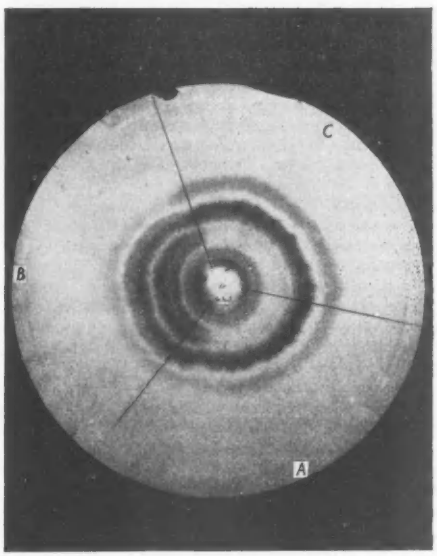

Fig. 2. Circular paper chromatogram showing the actinomycin mixtures produced by *Streptomyces antibioticus*. Solvent system: 10% aqueous solution of sodium *o*-cresotinate–di-*n*-butyl ether–*s*-tetrachloroethane (4:3:1, by vol.). Sequence of actinomycins is from centre to periphery; in all cases, the first zone just beyond the origin constitutes biologically-inactive coloured material. *A*, Actinomycin mixture produced in the glutamic acid–galactose medium; actinomycins I. II (trace), III (trace), IV and V; *B*, actinomycin mixture produced in the glutamic acid–galactose medium plus sarcosine (250 μg./ml.); actinomycins I, II, III, IV, an unidentified component and V; *C*, actinomycin mixture produced in the glutamic acid–galactose medium plus L-valine (1000 μg./ml.); same as in *A*.

Extraction of actinomycin. After a given period of incubation, the actinomycin in 500 ml. of a fermentation broth from a replicate series of flasks was extracted three times with butan-1-ol [20, 10, and 5% (v/v) respectively]. The extracts were combined and the butan-1-ol was removed by distillation *in vacuo*. The crude actinomycin residue was recovered in a small volume of acetone and used directly for circular paper chromatography. If necessary, the material could be recovered by evaporation of the acetone and then stored in the dry state in the dark until used.

Extraction of actinomycin from the mycelium of *S. antibioticus* was accomplished in the following manner: the mycelium in a sample of broth was collected by suction filtration and washed with distilled water until the wash water was free of antibiotic. The mycelium was then suspended in a small volume of Sørensen's M/15-phosphate buffer, pH 7·0, placed in a Raytheon 50 W, 9 k-cyc. magnetostrictor oscillator (Raytheon Manufacturing Co., Waltham, Mass., U.S.A.) and disrupted by treatment for 15–20 min. The actinomycin in the cell-free material was then treated as previously described.

Chromatographic separation of actinomycin mixtures. Chromatographic separation of actinomycin mixtures was accomplished by a circular-paper technique (Roussos & Vining, 1956; Katz, Pienta & Sivak, 1958). The solvent system consisted of equal volumes of a 10% (w/v) aq. solution of sodium *o*-cresotinate or *m*-cresotinate and a mixture of di-*n*-butyl ether and *s*-tetrachloroethane (3:1, v/v). The solvent system containing *o*-cresotinate was used for most of the experiments.

Determination of the percentages of components in an actinomycin mixture. The determination of the percentages of components in an actinomycin mixture was carried out spectrophotometrically (Goss & Katz, 1957). The effects observed with sarcosine represent shifts in synthetic patterns. As a consequence, increased formation of actinomycins II and III is accompanied by decreased production of actinomycin IV. The total yield of actinomycin remains essentially the same, however.

Production of actinomycin by washed suspensions of S. antibioticus. The organism was first grown in N-Z amine medium and a mycelial suspension was prepared. A portion of the washed mycelium was then inoculated into flasks of glutamic acid–galactose medium and allowed to grow for a 48 hr. period. The mycelium was harvested by centrifuging, washed twice in 0·09% NaCl soln. and finally suspended in water; the final amount of mycelium was 8 mg./ml. A 20 ml. amount of this suspension was inoculated into each of a duplicate series of 250 ml. Erlenmeyer flasks containing 20 ml. of M/15-phosphate buffer, 30 ml. of water and 10 ml. of an aqueous solution of sarcosine or L-glutamic acid (final concentration, 1 mM). In place of substrate, 10 ml. of water was added to the control flasks. The flasks were shaken at 240 rev./min. at 28°. The antibiotic titre of culture filtrates was determined daily by the disk-assay method. After 72 hr. incubation the actinomycin mixture was extracted from culture fluids and the percentage of components in the mixture was determined. No appreciable increase in cell weight occurred during the incubation period. Sterile conditions were maintained throughout the experiment.

RESULTS

Actinomycin synthesis in the presence and absence of sarcosine. The results of a study of actinomycin formation by *S. antibioticus* in the glutamic acid medium with and without sarcosine are presented in Figs. 3 and 4. Production of actinomycin mixtures began 24 hr. after inoculation of the organism and continued until the tenth day. Without sarcosine, actinomycin V was the major component produced initially, whereas actinomycin IV represented the chief constituent in the later stages of synthesis; actinomycins II and III were produced in trace amounts. With sarcosine present, actinomycins II and III constituted major components of the mixture within one day of its addition; moreover, the percentage of these substances in the mixture increased to some extent during the production period.

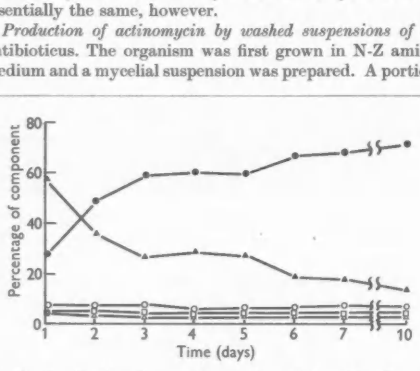

Fig. 3. Synthesis of an actinomycin mixture by *Streptomyces antibioticus* in glutamic acid medium. Five samples (500 ml.) of fermentation medium were taken daily, the actinomycin was extracted and the percentage of the components in a mixture determined. ○, Actinomycin I; △, actinomycin II; □, actinomycin III; ●, actinomycin IV; ▲, actinomycin V.

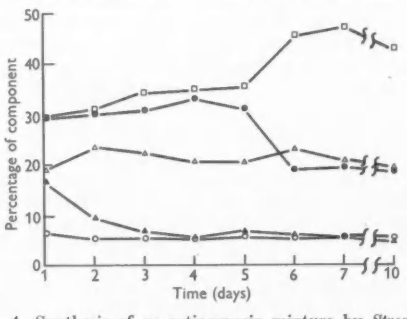

Fig. 4. Synthesis of an actinomycin mixture by *Streptomyces antibioticus* in glutamic acid medium plus sarcosine (250 μg./ml.). Sarcosine was added after 34 hr. incubation. Five samples (500 ml.) of fermentation medium were taken daily, the actinomycin was extracted and the percentage of the components in a mixture determined. ○, Actinomycin I; △, actinomycin II; □, actinomycin III; ●, actinomycin IV; ▲, actinomycin V.

Concentration of sarcosine. In Table 1 are presented data from a study in which different concentrations of sarcosine were added to the glutamic acid medium. A two- to three-fold increase in the amount of actinomycin III synthesized was observed when a concentration of 2·5 μg. of sarcosine/ml. was employed. Optimum formation of actinomycin II and III occurred, however, when sarcosine was supplied at concentrations of 250–500 μg./ml.

Nature of the actinomycin mixture produced in mycelium. Under the different nutritional conditions employed, it was of interest to determine whether the actinomycin mixture present in the mycelium of *S. antibioticus* was similar to or different from that found in the culture medium. Approximately 5–10% of the actinomycin synthesized was present in the mycelium; in addition, the actinomycin mixture was qualitatively and quantitatively similar to that found in the broth. Actinomycins II and III represented 35–40% of the mycelial actinomycin when the organism was grown in the presence of sarcosine; only trace quantities of these components could be found in cells grown in the absence of this amino acid.

Specificity of sarcosine. Compounds related to sarcosine biochemically or structurally were examined to ascertain whether they might influence synthesis of actinomycin in a similar manner. Choline, betaine, dimethylglycine, DL-phenylsarcosine, sarcosine anhydride, glycine, *N*-acetylglycine, glycine anhydride, glycylglycine, L-serine and L-methionine at concentrations ranging from 0·001 to 0·20% were tested. The addition of glycine, choline, betaine and dimethylglycine, at concentrations of 0·1–0·2%, increased the synthesis of actinomycin III approximately three- to four-fold; however, certain unrelated substances, e.g. L-threonine and L-aspartic acid, at similar concentrations produced the same effect. In no case was there increased formation of actinomycin II.

Influence of time of addition of sarcosine. Initially sarcosine was added to growing cultures at the onset of actinomycin production. To determine its effect on actinomycin formation at different times during synthesis, sarcosine (250 μg./ml.) was added to a series of cultures growing in the glutamic acid medium at 0, 1, 3, 5, 7 and 9 days after inoculation. At these periods, except for the initial set, the actinomycin mixture synthesized was extracted from a parallel series of cultures and the percentage of the components in a given mixture determined. After 10 days' incubation all experimental flasks were harvested and the nature of the actinomycin mixture in each set was determined. Thus the composition of the mixture produced before and after the addition of sarcosine was established.

Sarcosine was most effective when supplied during the early phases of actinomycin formation (Table 2). Optimum synthesis of actinomycins II and III occurred when sarcosine was added 24 hr. after inoculation of the organism—the point at which production of the antibiotic was just starting. When incorporated at the time of inoculation, it was almost as effective; subsequently the influence of sarcosine diminished as synthesis of the antibiotic decreased.

Single competing with multiple additions of sarcosine. Since the sarcosine (250 μg./ml.) fed as a single addition disappeared from the medium within 2–3 days of its incorporation, it was likely that part of the exogenous supply was metabolized

Table 1. *Influence of sarcosine concentration on actinomycin biosynthesis*

Sarcosine was added to glutamic acid medium after 30 hr. incubation of *S. antibioticus*. After an additional 6 days' cultivation, the actinomycin mixture synthesized was harvested by extraction with butan-1-ol. After removal of the butanol the actinomycin mixture was separated by means of circular paper chromatography, and the percentage of actinomycin components in a mixture determined by a spectrophotometric method (Goss & Katz, 1957).

Concn. of sarcosine (μg./ml.)	Percentage of actinomycin components				
	I	II	III	IV	V
0·0	6·2	2·1	2·8	80·4	8·4
2·5	6·5	1·9	7·3	70·8	13·6
10·0	5·3	9·2	11·9	63·5	10·1
50·0	6·5	21·2	30·8	35·9	5·6
100·0	8·1	22·5	33·1	30·8	5·5
250·0	8·9	25·4	35·3	24·6	5·7
500·0	9·1	25·6	35·0	24·4	5·9

Table 2. *Influence of time of addition of sarcosine on synthesis of actinomycins II and III*

Sarcosine (250 μg./ml.) was added to glutamic acid medium at the times indicated. Actinomycin mixtures were harvested on the tenth day of cultivation; final antibiotic titre 101 μg. of actinomycin/ml. The data represent the percentages of actinomycins II and III in the sarcosine-stimulated actinomycin mixture *less* the percentage of these actinomycins in the actinomycin mixture produced in the absence of sarcosine.

Age of culture at time of sarcosine addition (days)	Actinomycin titre at time of sarcosine addition (μg./ml.)	Percentage of components after 10 days' incubation	
		Actinomycin II	Actinomycin III
0	0	23·7	25·9
1	1	26·4	32·6
3	23	16·3	27·1
5	73	11·7	18·8
7	88	6·9	13·1
9	92	0·0	4·1

by the organism. Consequently the maximum effect possible with this compound could not be attained. To compensate for the metabolic loss, attempts were made to increase the level of sarcosine in the medium. When given as a single addition, however, it was evident that levels beyond the optimum concentration noted previously were somewhat inhibitory to antibiotic synthesis, the final antibiotic titre being greatly decreased without any appreciable increase in the amount of actinomycin II and III formed. A given concentration of the amino acid was slightly but consistently more effective, however, when given in small portions over several days.

Production of actinomycin by washed suspensions of S. antibioticus. The study carried out with washed suspensions of *S. antibioticus* should be regarded as preliminary since many experimental details require further investigation. For example, the actinomycin titre in control flasks was approximately the same as that in the experimental groups. Similar results were obtained initially in studies on penicillin formation by washed suspensions of *Penicillium chrysogenum* (Demain, 1956). This effect was attributed to the high endogenous reserves in the mycelium which the organism used for production of the antibiotic. The differences observed in the nature of the actinomycin mixture synthesized in the presence and absence of sarcosine, however, appear to be significant.

In the absence of substrate, *S. antibioticus* produced approximately equal amounts of actinomycins IV and V; with glutamate, actinomycin IV was the chief constituent, representing 52 % of the actinomycins formed. When sarcosine was employed, actinomycin III increased from 5·3 to 27·3 % of the mixture synthesized. No change occurred in the amount of actinomycin II formed under these conditions.

Reversal of sarcosine effect by L-*proline.* Exogenous sarcosine may influence synthesis of actinomycin mixtures either directly, by its incorporation into certain actinomycin peptides, or indirectly, by modifying the metabolism and consequently the antibiotic-producing activities of the organism. The data obtained in this investigation suggest that exogenous sarcosine is directly incorporated into actinomycins II and III by competing with endogenous forms of proline for the 'proline' site in certain actinomycin peptides. Actinomycin II contains no proline, and actinomycin III only 1 mole of proline. Both possess additional sarcosine (Johnson & Mauger, 1959). If sarcosine does compete with proline and replaces it in certain peptides, it should be possible to reverse the effect of sarcosine by supplementing endogenous proline with an exogenous source. Conversely, it should be possible to abolish the activity of the added proline by increasing the concentration of sarcosine in the medium.

The results of a series of experiments provide evidence in support of these conclusions (Table 3). Suitable concentrations of L-proline considerably diminished the effect of sarcosine. When an additional concentration of sarcosine was added to the medium, exogenous proline was no longer able to reverse the effect of sarcosine to any appreciable extent.

Addition of DL-*pipecolic acid.* Additional support for the hypothesis that sarcosine competes with proline was obtained by using an analogue of proline, DL-pipecolic acid. This compound might also compete because of its close structural relationship to proline. If this were so, new actinomycins containing pipecolic acid or its derivatives might be formed.

According to the concentrations of DL-pipecolic acid employed, 1–5 new actinomycins were formed by *S. antibioticus*. Fig. 5 shows the composition of the mixture produced with 100, 250, 500, 1000 and 2000 μg. of DL-pipecolic acid/ml. in the medium. These new compounds represented approximately 48 % of the actinomycin mixture synthesized. In addition, by paper chromatography of acid hydrolysates, pipecolic acid was shown to be present in certain of the new actinomycin components.

Because of the close similarity between proline and pipecolic acid, it appeared likely that the analogue would compete more favourably with endogenous proline than sarcosine for the 'proline' site. The results of an experiment confirmed this view (Table 4). When simultaneous additions of sarcosine and pipecolic acid were made, the effect of sarcosine decreased as the concentration of DL-pipecolic acid increased.

Table 3. *Percentage of components II and III in actinomycin mixtures synthesized in the presence of sarcosine and* L-*proline*

Sarcosine and proline were added to glutamic acid medium after 24 hr. incubation. After 6 more days' cultivation the actinomycin mixture synthesized was harvested and the percentage of components determined.

Concn. of sarcosine (μg./ml.)	Concn. of L-proline (μg./ml.)	Actinomycins II and III (%)	Actinomycins I, IV and V (%)
Expt. 1			
100	0	62·4	37·6
100	500	59·2	40·8
100	1000	44·6	55·4
100	2000	18·2	81·8
Expt. 2			
500	0	57·0	43·0
500	500	55·5	44·4
500	1000	51·2	49·8
500	2000	47·1	53·0

DISCUSSION

It appears likely that an exogenous amino acid (*a*) is incorporated directly into certain actinomycin peptides, or (*b*) is first partly modified by the organism before its incorporation, or (*c*) acts indirectly by altering the metabolic activities of the organism and subsequently influencing antibiotic synthesis as well. We shall first consider the mechanism of synthesis of an actinomycin mixture under the normal conditions of cultivation.

Johnson (1956) suggested that an actinomycin molecule is formed through the oxidative condensation of 2 molecules of 3-hydroxy-4-methylanthranilic acid peptide. The peptides involved may or may not be identical. If the anthranilic acid peptide is the final intermediate assembled before synthesis of a molecule of the antibiotic, some specific enzymic site must exist in the cell to synthesize this compound. The structural units of the antibiotic (amino acids, 3-hydroxy-4-methylanthranilic acid), possibly formed elsewhere in the cell, must be transported to this enzymic site. There, a process comparable with patternization may occur (Gale, 1958) so that the various molecules are arranged in proper sequence before the formation of bonds between adjacent amino acid residues and the anthranilic acid moiety. It appears reasonable to assume that such a specific synthetic site is involved and that some kind of sequential arrangement of the amino acid residues and the anthranilic acid moiety precedes synthesis of the 3-hydroxy-4-methylanthraniloyl peptide.

The amount of anthranilic acid peptide formed would depend, in part, on the presence of each of the amino acids and 3-hydroxy-4-methylanthranilic acid at this site at the appropriate time. A lack of one of the constituents would result in little, if any, synthesis; a delay in appearance of one of the compounds would limit synthesis of the anthranilic acid peptide and ultimately, of course, of actinomycin.

Since the only difference in the actinomycin peptides produced by *S. antibioticus* occurs at the 'proline' position, it is likely, under conditions of synthesis, that competition exists between proline, hydroxyproline, 4-oxoproline and, to a slight extent, sarcosine for the 'proline' spot on the surface of this specific enzymic site. If there exists a greater affinity for proline than for any of

Table 4. *Influence of sarcosine and* DL-*pipecolic acid on the synthesis of actinomycins II and III*

Sarcosine and DL-pipecolic acid were added to glutamic acid medium after 36 hr. incubation. On the seventh day of cultivation the actinomycin mixture synthesized was harvested and the percentage of components determined.

Concentration (μg./ml.) of		Percentage of components in actinomycin mixture	
DL-Pipecolic acid	Sarcosine	Actinomycins II and III	Pipecolic acid components
0	0	6·8	0
0	250	67·2	0
5	250	62·7	5·4
10	250	57·1	14·4
25	250	38·8	18·9
100	250	16·2	44·5
100	0	6·1	48·2

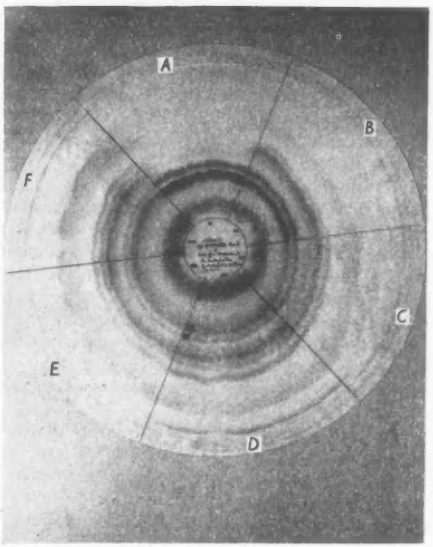

Fig. 5. Circular paper chromatogram showing the actinomycin mixtures produced by *Streptomyces antibioticus*. Solvent system: 10% aqueous solution of sodium *o*-cresotinate–di-*n*-butyl ether–*s*-tetrachloroethane (4:3:1, by vol.). Sequence of actinomycins is from centre to periphery; actinomycin I has not emerged from the first biologically-inactive coloured zone just beyond the origin. In all cases it appears that actinomycins I to V were formed. *A*, Glutamic acid–galactose medium; actinomycins I to V and a sixth unidentified component; *B*, glutamic acid–galactose medium plus DL-pipecolic acid, 100 μg./ml.; actinomycins I–V and three additional components; *C*, glutamic acid–galactose medium plus DL-pipecolic acid, 250 μg./ml.; actinomycins I–V and four additional components; *D*, glutamic acid–galactose medium plus DL-pipecolic acid, 500 μg./ml.; actinomycins I–V and five additional components; *E*, glutamic acid–galactose medium plus DL-pipecolic acid, 1000 μg./ml.; actinomycins I–V and four additional components; *F*, glutamic acid–galactose medium plus DL-pipecolic acid, 2000 μg./ml.; actinomycins I–V and four additional components.

the other compounds mentioned, or if the concentration of proline at this site is greater than that of the other substances, we can expect it to be incorporated into the actinomycin peptides much more frequently than are the other amino acids. When condensation of two molecules of anthranilic acid peptide occurs, it should take place most frequently with peptides containing proline (actinomycin IV, 60–80%) (Goss & Katz, 1957). Condensation of a peptide containing proline with one containing hydroxyproline (actinomycin I, 5–8%), 4-oxoproline (actinomycin V, 5–15%) or sarcosine (actinomycin III, 3–4%) will occur much less frequently. Condensations of two peptides with hydroxyproline or 4-oxoproline or sarcosine (actinomycin II, 2–3%) probably take place also, but the frequency of this event would be of a very low order. Components produced in such trace amounts would be extremely difficult to detect with the chromatographic systems available at present.

If one modifies the nutritive environment, e.g. by changing the nitrogen source (Katz, Pienta & Sivak, 1958), adding a second one (Schmidt-Kastner, 1956*a*, *b*; Katz & Goss, 1958) or changing the C/N ratio (Martin & Pampus, 1956), different equilibria may arise between the competing amino acids of a given system. In this newly established condition a different actinomycin component may become the major one of a mixture or it may at least increase in amount relative to the other components.

If one introduces an exogenous supply of one of the amino acids in a competing system, the opportunity to shift the equilibrium becomes even greater, as was observed with hydroxyproline and actinomycin I (Katz & Goss, 1958), sarcosine and actinomycins II and III with *S. antibioticus*, and DL-valine and actinomycin IV with *S. chrysomallus* (Schmidt-Kastner, 1956*b*). An exogenous compound may be altered first by the organism; the modified compound then competes with a naturally synthesized amino acid for a particular site in the peptide. For example, *S. chrysomallus* converts isoleucine into *N*-methylisoleucine and the methylated amino acid competes with endogenous *N*-methylvaline (Schmidt-Kastner, 1956*a*, *b*). The condensation of an anthranilic acid peptide containing the former amino acid with a natural one, or the condensation of two such peptides, would result in synthesis of new actinomycins.

Finally, the addition of a substance closely related to an amino acid found in actinomycin may result in competition between the endogenously synthesized substance and the exogenous compound; incorporation of such related substances into the peptides will probably occur with new actinomycins ultimately synthesized. Pipecolic acid appears to be incorporated directly into actinomycin molecules in this manner.

To what extent can the actinomycin peptides be modified? Can one, for example, replace any one of the amino acids in these peptides? Can one replace two or more amino acids simultaneously? Also, what is the specificity of the various amino acid receptors present on the surface of the peptide-synthesizing site? The information available concerns chiefly the 'proline' position in the various peptides; it appears that the specificity requirements of the 'proline' receptor site are not of a particularly high order. In some cases the exogenous compound bears a close structural relationship to the endogenous substance it competes with (hydroxyproline, pipecolic acid competing with proline); in other instances, only a portion of the exogenous molecule is found to be similar to a fragment of the natural amino acid (sarcosine competing with proline). Since proline, hydroxyproline, oxoproline, pipecolic acid and sarcosine probably all compete for the same location in the peptide, a compound must have the structural configuration —$H_2C{\cdot}NH{\cdot}CH(CO_2H)$— to compete successfully with proline for this position. It is possible to predict, therefore, that *N*-methyl amino acids, proline analogues and certain substituted forms of these substances should also compete favourably with proline.

The number of new compounds synthesized in any given situation will vary with the amino acid, the concentration used, the affinity of the surface of the specific synthesizing site etc. On theoretical grounds, one would expect several new compounds to be produced in each instance where incorporation takes place. For example, with pipecolic acid it should be possible to find actinomycin peptides containing (1) pipecolic acid/pipecolic acid, (2) proline/pipecolic acid, (3) hydroxyproline/pipecolic acid and (4) oxoproline/pipecolic acid. In addition, isomers of the actinomycins containing unsymmetrical peptides are probably synthesized. For sarcosine, one should find sarcosine/sarcosine and sarcosine/proline peptides and, in addition, sarcosine/hydroxyproline and sarcosine/ketoproline peptides. Isomers of the last three combinations should also exist.

Controlled biosynthesis can be defined as the technique of predetermining the structure of a new antibiotic by furnishing specific chemical precursors to the antibiotic-producing micro-organism (Woodruff & McDaniel, 1958). This technique has been successfully applied to the formation of modified forms of actinomycin. It should be possible to utilize this procedure with other antibiotic-producing micro-organisms for developing antibiotics with new or changed properties.

SUMMARY

1. Increased synthesis of actinomycins II and III occurred when sarcosine was added to the medium of growing cultures of *Streptomyces antibioticus*. The amount of actinomycins II and III formed depends, in part, on the concentration of sarcosine and on the time and the number of additions of this amino acid. Mycelial actinomycin, as well as that produced in the medium, contains considerable amounts of actinomycins II and III.

2. The effect observed with sarcosine is highly specific; compounds structurally and biochemically related were ineffective.

3. Exogenous L-proline reversed the effect of a given concentration of sarcosine; larger amounts of sarcosine abolished the activity of proline.

4. Incorporation of DL-pipecolic acid, a proline analogue, into the medium resulted in synthesis of several new actinomycins.

5. When washed suspensions of *S. antibioticus* were incubated in the presence of 1 mM-sarcosine, there was a fivefold increase in synthesis of actinomycin III, but no change in the percentage of actinomycin II.

6. The results obtained support the view that exogenous sarcosine and pipecolic acid compete with and replace endogenous proline in certain actinomycin peptides.

7. A generalized theory of actinomycin biogenesis is proposed.

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The Formation of Mercapturic Acids

3. *N*-ACETYLATION OF *S*-SUBSTITUTED CYSTEINES IN THE RABBIT, RAT AND GUINEA PIG*

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In the previous papers (Barnes, James & Wood, 1959; Bray, Franklin & James, 1959) evidence was given which supported the hypothesis that, in the formation of mercapturic acids *in vivo*, glutathione and glutathionase are involved in the synthesis of *S*-substituted cysteines. The subsequent acetylation of these compounds to mercapturic acids remained to be examined. During this work it was observed that, although guinea-pig liver had greater glutathionase activity than had the livers of either the rabbit or the rat, the guinea pig excreted unexpectedly small amounts of mercapturic acids when given compounds which were readily converted into mercapturic acids by the rabbit and rat. A possible explanation of the failure of the guinea pig to excrete mercapturic acid

* Part 2. Bray, Franklin & James (1959).

would be a failure to acetylate *S*-substituted cysteines. This provided an additional reason for studying the acetylation stage in the synthesis of mercapturic acid with special reference to the guinea pig. Several workers have investigated the acetylation of *S*-substituted cysteines in the rat, e.g. Stekol (1938), Zbarsky & Young (1943), West, Mathura & Black (1951) and West & Mathura (1954), but there is little available information about the rabbit and none for the guinea pig.

While much work has been done on the acetylation of aromatic amino compounds *in vitro*, there have been few investigations of *N*-acetylation in aliphatic compounds. Gutmann & Wood (1951) reported the acetylation by rat liver and kidney slices of *S*-benzyl-DL-homocysteine and *S*-(*p*-bromophenyl)-L-cysteine. Chou & Soodak (1952) prepared extracts of pigeon liver which acetylated glucosamine and chondrosamine. Davidson, Blumental & Roseman (1957) showed that rabbit liver, kidney and muscle, dog kidney and human liver acetylated glucosamine 6-phosphate. Phillips & Anker (1957) showed that both rat liver and kidney could acetylate 3-*cyclo*hexyl-L-alanine. The acetylation of some *S*-substituted cysteines and some other amino compounds has now been investigated *in vivo* and in liver and kidney slices of the rabbit, rat and guinea pig and the results suggest that, in general, the guinea pig does not acetylate aliphatic amino groups as readily as does the rabbit or the rat.

MATERIALS

All melting points are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn. Chlorine in *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine was determined by the flask method (see Macdonald, 1959) and titration with silver nitrate and dithizone (Archer, 1958).

Chlorobenzene, benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane, 3:4-dichloronitrobenzene, 2:3:5:6-tetrachloronitrobenzene, D-glucosamine and *p*-aminobenzenesulphonamide were purchased. *N*-Acetyl-D-glucosamine, $[\alpha]_D^{23}$ in water $+38\pm5°$ (*c*, 0·5), was prepared by acetylation of D-glucosamine with silver acetate (cf. Stacey, 1944). *S*-(*p*-Chlorophenyl)-L-cysteine, m.p. 193°, was prepared according to Parke & Williams (1951*a*). The other *S*-substituted cysteines were prepared from halogeno compounds and cysteine by a modification of the method of Suter (1895) as described by Bray, James & Thorpe (1958), who described the properties of *S*-benzyl-L-cysteine and *S*-(*p*-chlorobenzyl)-L-cysteine. *S*-(*2-Chloro-4-nitrophenyl*)-L-*cysteine* formed from aqueous ethanol pale-yellow needles, m.p. 182°, turning brown when kept in bright daylight; $[\alpha]_D^{23}+11\pm5°$ in 0·1 N-KOH (*c*, 0·5); light-absorption max. in 0·1 N-KOH 347 mμ (ϵ 9382), min., 275 mμ (Found: C, 39·4; H, 3·5; N, 9·7; Cl, 12·3; S, 11·1. $C_9H_9O_4N_2ClS$ requires C, 39·1; H, 3·3; N, 10·1; Cl, 12·8; S, 11·6%). *S*-Butyl-L-cysteine formed from aqueous ethanol colourless needles, m.p. 246° (decomp.); $[\alpha]_D^{23}+24\pm5°$ in 0·1 N-KOH (*c*, 0·8) (Found: C, 47·7; H, 7·8; N, 8·0; S, 18·5. Calc. for $C_7H_{15}O_2NS$: C, 47·4; H, 8·5; N, 7·9; S, 18·1%). Armstrong & Lewis (1951) give m.p. 242–244° (decomp.) and Stoll & Seebeck (1949) 220–230° (decomp.). *S*-(2:3:5:6-*Tetrachlorophenyl*)-L-*cysteine* formed from absolute ethanol colourless whetstones, m.p. 179°; $[\alpha]_D^{23}$ in 0·1 N-KOH $0\pm7°$ (*c*, 0·7); light-absorption max. in 0·1 N-KOH 287 mμ (ϵ 2480) (Found: C, 32·7; H, 2·1; N, 4·4; Cl, 42·0; S, 9·4. $C_9H_7O_2NCl_4S$ requires C, 32·2; H, 2·1; N, 4·2; Cl, 42·4; S, 9·6%). The mercapturic acids required as reference compounds were prepared by acetylation of the *S*-substituted cysteines with acetic anhydride and NaOH as described by Zbarsky & Young (1943). *N*-*Acetyl*-*S*-*butyl*-L-*cysteine* formed from ether–light petroleum (b.p. 40–60°) colourless prisms, m.p. 67°; $[\alpha]_D^{23}-24\pm3°$ in ethanol (*c*, 0·6) (Found: C, 49·6; H, 7·9; N, 6·6; S, 14·8%; equiv. 204. $C_9H_{17}O_3NS$ requires C, 49·3; H, 7·8; N, 6·4; S, 14·6%; equiv. 219). The properties of the other mercapturic acids used have already been described (e.g. Jaffe, 1879; Spencer & Williams 1950; Stekol, 1938; Bray *et al.* 1958; Bray, James & Thorpe, 1957; Betts, James & Thorpe, 1955; Bray, Hybs, James & Thorpe, 1953).

METHODS

Animals, diet and dosage

The rabbits (about 2·5 kg.) used and their diet were as previously described (Bray, Ryman & Thorpe, 1947). Guinea pigs (about 500 g.) had the same diet and rats (about 200 g.) were maintained on a diet of rat cubes and water *ad lib.* The compounds were administered with water by stomach tube. No toxic effects were observed except with *S*-(2-chloro-4-nitrophenyl)-L-cysteine in the guinea pig. This compound (dose 72 μmoles/100 g.) caused severe anorexia and death ensued after about 7 days. 3:4-Dichloronitrobenzene (dose 104 μmoles/100 g.) also caused some anorexia in the guinea pig but the animals recovered in 3 days.

Preparation of tissue slices

The animals were killed by a sharp blow on the back of the neck. The organs required were removed rapidly and kept in ice-cold Ringer phosphate buffer, pH 7·4, prepared according to Umbreit, Burris & Stauffer (1949). Tissue slices (thickness 0·2–0·4 mm.) were cut between ground-glass plates by a razor blade and transferred to ice-cold Ringer phosphate solution. When required they were dried on filter paper, then weighed (1–2 g.) into a conical flask containing 10 ml. of a solution of substrate and glucose in Ringer phosphate buffer which had previously been saturated with oxygen. The reaction mixture was incubated at 37° with continuous shaking.

Determination of metabolites in urine

Mercapturic acids were determined by the methods recorded by Bray *et al.* (1958) and Barnes *et al.* (1959). The validity of the determinations in the presence of the corresponding *S*-substituted cysteines (see Table 3) requires comment. *S*-Benzyl-L-cysteine and *S*-(*p*-chlorobenzyl)-L-cysteine do not react under the conditions used in the nitroprusside method for determining the mercapturic acids. The treatment with zinc hydroxide in the iodometric method entirely removes added *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine from urine. The intensity of the yellow colour given by *S*-(2-chloro-4-nitrophenyl)-L-cysteine under the conditions for the colorimetric method is

Table 1. *R_F values and conditions for identification of metabolites by paper chromatography*

Solvent mixtures were: *A*, butan-1-ol–water–acetic acid–butyl acetate (24:10:5:2, by vol.); *B*, butan-1-ol saturated with aq. 2N-NH_3 soln.; *C*, benzene–acetic acid–water (2:2:1, by vol.). The descending method was used, unless stated otherwise, on Whatman no. 4 or 3MM paper.

Compound	R_F value	Time of run (hr.)	Solvent mixture
S-Butyl-L-cysteine	0·73	7	*A*
	0·35	16	*B*
N-Acetyl-*S*-butyl-L-cysteine	0·92	7	*A*
	0·46	16	*B*
Unidentified metabolite of bromobutane	0·20*	16	*B*
S-(2:3:5:6-Tetrachlorophenyl)-L-cysteine	0·60*	14	*A*
	0·44	16	*B*
N-Acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine	0·95	14	*A*
	0·60	16	*B*
Unidentified metabolite of *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine	0·13	14	*A*
	0·05	16	*B*
2:3:5:6-Tetrachlorothiophenol	0·75†	4	*B*
	1·0†	4	*C*
S-(2-Chloro-4-nitrophenyl)-L-cysteine	0·5‡	16	*A*
S-Benzyl-L-cysteine	0·63	7	*A*
	0·22	16	*B*
N-Acetyl-*S*-benzyl-L-cysteine	0·93	7	*A*
	0·41	16	*B*

* Streaks. † Ascending method. ‡ Decomposes in solvent *B*.

less than 10% of that given by the mercapturic acid. The determination of the mercapturic acids corresponding to these four *S*-substituted cysteines can be regarded as valid.

Under the more drastic conditions of hydrolysis used for determination of butylmercapturic acid by the nitroprusside method (Barnes *et al.* 1959) *S*-butylcysteine gave about 50% of the colour given by an equivalent amount of butylmercapturic acid. Treatment of rabbit, rat or guinea-pig urine with zinc hydroxide (as in the iodometric method) before the alkaline hydrolysis caused a loss of added butylmercapturic acid not exceeding 10%, but with *S*-butylcysteine the loss was 40 ± 10% from rabbit urine and 70 ± 5% from rat and guinea-pig urine. Determinations by the nitroprusside method with and without treatment with zinc hydroxide should therefore reveal the presence of appreciable amounts of *S*-butylcysteine in urine. By the iodometric method only 12% of added *S*-(*p*-chlorophenyl)-L-cysteine was recovered from urine treated with zinc hydroxide so that the presence of small amounts of this compound would not influence the result for *N*-acetyl-*S*-(*p*-chlorophenyl)-L-cysteine significantly.

Ethereal sulphate was determined by the method of Folin (1905–06). Glucuronic acid was determined by the modified naphthoresorcinol method described by Bray, Humphris, Thorpe, White & Wood (1952). Anilines were determined as described by Bray, James & Thorpe (1956).

Determination of unabsorbed material

To eliminate the possibility that low results might be caused by poor absorption of the compounds administered, guinea-pig faeces were examined for unabsorbed material where a method was available. 3:4-Dichloronitrobenzene and 2:3:5:6-tetrachloronitrobenzene were determined in the steam-distillate of the faeces as described by Bray *et al.* (1953, 1956). The 24 hr. samples of faeces passed immediately before the dose of *S*-(*p*-chlorophenyl)-L-cysteine and for the 2 days afterwards were separately finely ground and extracted with 500 ml. of 0·1N-NaOH. The light-absorption of a centrifuged sample of the extract, suitably diluted, was measured. A calibration curve was constructed by measuring the difference in the absorption at 258 and 310 mμ of solutions made by the addition of known amounts of *S*-(*p*-chlorophenyl)cysteine to a similar extract of normal faeces. The same method was used for *S*-(2-chloro-4-nitrophenyl)-L-cysteine except that the absorptions were measured at 350 mμ and 410 mμ.

Paper chromatography

The procedure was as described by Bray *et al.* (1956, 1957). Additional solvent mixtures used and R_F values are given in Table 1. Mercapturic acids and *S*-substituted cysteines were detected by the potassium dichromate–silver nitrate reagent of Knight & Young (1958) and by ninhydrin.

Determination of acetylation by tissue slices

p-*Aminobenzenesulphonamide as substrate.* A sample (1 ml.) of the medium was removed and deproteinized by the addition of 10% (w/v) $ZnSO_4,7H_2O$ (5 ml.) and 0·5N-NaOH (5 ml.). The precipitate was removed by centrifuging and 2 ml. of the supernatant was added to 3 ml. of water and the amino compounds were determined by the method of Bratton & Marshall (1939). The intensity of colour was measured in a Spekker photoelectric absorptiometer with a Chance glass filter OG1.

D-*Glucosamine as substrate.* The procedure of Morgan & Elson (1934) was adapted. The mixture of slices and medium was homogenized with a Potter–Elvehjem type homogenizer with a polytetrafluoroethylene (Teflon) pestle. Duplicate samples (4 ml.) of the homogenate were

mixed with absolute ethanol (5 ml.) and filtered after 30 min. Samples (2 ml.) of the filtrate were transferred to test tubes graduated at 10 ml. Then 0·1 ml. of 0·5 N-Na_2CO_3 was added and the mixture was heated in a boiling-water bath for exactly 5 min. The tube was fitted with a 'cold finger' to reduce evaporation. To the cooled mixture 6 ml. of acetic acid and 1 ml. of *p*-dimethylaminobenzaldehyde reagent (2 g. in 95 ml. of acetic acid and 5 ml. of conc. HCl) were added. The volume was made up to 10 ml. with acetic acid and the mixture was kept in the dark for not less than 45 min. before the extinction was measured in a Spekker photoelectric colorimeter with a Chance filter OG1. Calibration curves were prepared from *N*-acetyl-D-glucosamine in water. The average recovery of *N*-acetyl-D-glucosamine added to liver slices in six control experiments was 71% (range 66–75%). This method is more sensitive than that described by Chou & Soodak (1952); see Table 2.

S-(p-Chlorobenzyl)-L-cysteine and S-butyl-L-cysteine as substrates. The mixture of slices and medium was homogenized as described above and 0·5% uranyl acetate (10 ml.) was mixed with the homogenate. After 30 min. the mixture was filtered and 13 ml. of the filtrate was acidified (pH 1·0) with conc. HCl and continuously extracted with ether for 6 hr. The extract was concentrated to 5 ml., transferred to a boiling tube with three washings with 5 ml. of ether and evaporated to dryness. Water (5 ml.) was added and mercapturic acid was determined by the nitroprusside method described by Bray *et al.* (1958). The mean recovery of *p*-chlorobenzylmercapturic acid added to liver slices in six experiments was 68±4%. That for the butylmercapturic acid was 63% (range 56–68%).

Table 2. *Comparison of methods for determination of acetylglucosamine*

For experimental details see Chou & Soodak (1952) and the Methods section. The amounts of acetylglucosamine indicated were in 10 ml. of the final solution in the cuvette.

Acetylglucosamine present (μg.)	Spekker reading	
	Method of Chou & Soodak	Present method
500	0·215	0·505
400	0·165	0·430
250	0·105	0·312
100	0·03	0·150
50	0·01	0·085
0	0·01	0·01

RESULTS

Absorption of 3:4-dichloronitrobenzene, 2:3:5:6-tetrachloronitrobenzene, S-(p-chlorophenyl)-L-cysteine and S-(2-chloro-4-nitrophenyl)-L-cysteine by the guinea pig and rat

No 3:4-dichloronitrobenzene or 3:4-dichloroaniline was detected in the faeces excreted by two guinea pigs during 72 hr. after dosage with 3:4-dichloronitrobenzene. In two experiments with 2:3:5:6-tetrachloronitrobenzene 14 and 22% of the dose were found in the faeces. In similar experiments with rats the values were 19 and 28%. No unchanged material was detected in the faeces of two guinea pigs which had each received 75 mg. of *S*-(*p*-chlorophenyl)-L-cysteine; since the method of analysis would have detected 10% of the dose in the faeces, it is probable that at least 90% was absorbed provided that no breakdown of the compound took place in the alimentary canal. Analysis of the faeces of two guinea pigs dosed with *S*-(2-chloro-4-nitrophenyl)-L-cysteine failed to reveal

Table 3. *Acetylation of S-substituted L-cysteines by the rabbit, rat and guinea pig*

Results are expressed as percentages of the dose excreted as mercapturic acid and are given as means with ranges in parentheses; superior figures indicate the number of experiments. Samples of urine were collected until mercapturic acid could no longer be detected, usually 24 hr. For validity of results for *S*-*p*-chlorophenyl-L-cysteine, *S*-butyl-L-cysteine and *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine see Methods and Discussion.

Compound administered	Rabbit		Rat		Guinea pig	
	Dose (μmoles/100 g.)	Acetylation (% of dose)	Dose (μmoles/100 g.)	Acetylation (% of dose)	Dose (μmoles/100 g.)	Acetylation (% of dose)
S-(*p*-Chlorophenyl)-L-cysteine	173	51 (38–69)[6]	108	37 (34–42)[3]	65	4 (0–12)[3]
S-Benzyl-L-cysteine	95	45 (35–55)[3]	118	56 (52–62)[3]	71	13 (11–14)[3]
S-(*p*-Chlorobenzyl)-L-cysteine	82	43 (34–50)[3]	123	80 (65–100)[3]	73	3 (2–5)[3]
S-Butyl-L-cysteine	114	39 (30–47)[3]	142	61 (37–69)[8]	85	31* (22–43)[6]
S-(2-Chloro-4-nitrophenyl)-L-cysteine	72	62 (56–67)[4]	127	57 (47–63)[3]	72	9 (6–11)[3]
S-(2:3:5:6-Tetrachlorophenyl)-L-cysteine	—	—	75	9 (6–13)[3]	45	65* (42–90)[6]

* No mercapturic acid could be isolated from these urines and it is doubtful whether any of this acid was excreted (see text).

any unchanged material in one animal and gave 0·8 % of the dose in the second, so that subject to the above proviso this compound seems to be almost completely absorbed.

N-*Acetylation of* S-*substituted* L-*cysteines by rabbit, rat and guinea pig* in vivo

The results obtained are given in Table 3. From the urine of one rabbit 45 % of the dose of *S*-(2-chloro-4-nitrophenyl)-L-cysteine was isolated by the method of Barnes *et al.* (1959) as *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine, m.p. 191° alone and in admixture with an authentic specimen; light-absorption max. 345 mμ (ε 10 530). Similarly, from rat urine 17 % of the dose was isolated as the mercapturic acid, m.p. and mixed m.p. 190°; light-absorption max. 345 mμ (ε 10 700). No mercapturic acid was isolated from the urine of guinea pigs dosed with *S*-(2-chloro-4-nitrophenyl)-L-cysteine. Butylmercapturic acid was determined in the urines of animals given bromobutane or *S*-butylcysteine with and without treatment of the urine with zinc hydroxide. The agreement between the two determinations (Table 4) suggests that little, if any, *S*-butylcysteine is excreted.

Acetylation of p-*aminobenzene sulphonamide by the guinea pig* in vivo

To confirm that the guinea pigs used would acetylate an aromatic amine, three guinea pigs were each given 50 mg. of *p*-aminobenzene sulphonamide. Analysis of urine collected during the next 48 hr. showed that 25 ± 5 % of the dose was excreted as free *p*-aminobenzene sulphonamide and 56 ± 6 % of the dose was acetylated.

N-*Acetylation by tissue slices*

The results obtained for *N*-acetylation of amino compounds by tissue slices are summarized in Table 5. Since there is some doubt about the validity of the results obtained in the determination of butylmercapturic acid, experiments with liver slices similar to those of Table 5 were carried out with *S*-benzylcysteine and *S*-butylcysteine, in each of which the combined contents of three flasks (total 30 ml.) which had been incubated for 4 hr. were filtered through cotton wool. A sample of the filtrate was applied to Whatman no. 3MM paper and developed with solvent mixtures *A* and *B*. The remainder of the filtrate was acidified with HCl to pH 1·0 and continuously extracted with ether. The ether extract was examined by paper chromatography as described above. The unchanged cysteines were readily detected on the paper chromatograms from the filtrates of the liver slices of all three species. With *S*-benzylcysteine the mercapturic acid was readily detected in the ether extracts from liver slices of the rat and rabbit but only traces were present in those of the guinea pig. With *S*-butylcysteine the mercapturic acid was readily detected in the ether extracts from rat slices but only traces were found in those from the guinea pig and from the rabbit.

Metabolism of 3:4-*dichloronitrobenzene and* S-(2-*chloro*-4-*nitrophenyl*)-L-*cysteine in the guinea pig*

The metabolism of these compounds was of particular interest since *S*-(2-chloro-4-nitrophenyl)-L-cysteine, which would be expected to be an intermediary metabolite of 3:4-dichloronitrobenzene, was very toxic to the guinea pig. Quantitative analysis of 24 hr. urine of guinea pigs which had received 3:4-dichloronitrobenzene (104 μmoles/100 g.) showed the presence of mercapturic acid corresponding to an average of 3 % of the dose (range 2–5 % in five experiments), ethereal sulphate to 12 % (7, 17 %) and 3:4-dichloroaniline

Table 4. *Effect of zinc hydroxide treatment on results for butylmercapturic acid*

Mercapturic acid was determined by the nitroprusside method in urines with (B) and without (A) treatment with zinc hydroxide. Doses of bromobutane were 190, 175 and 108 μmoles/100 g. for rabbits, rats and guinea pigs respectively; those of *S*-butyl-L-cysteine were 114, 142 and 85 μmoles/100 g. respectively. Results are expressed as means with ranges in parentheses; superior figures indicate the number of experiments.

Compound administered	Treatment of urine	Mercapturic acid excreted (% of dose)		
		Rabbit	Rat	Guinea pig
Bromobutane	A	34 (30–37)^5	60 (47–72)^5	22* (19–28)^4
	B	32 (27–36)^5	57 (43–63)^5	18* (13–21)^4
S-Butyl-L-cysteine	A	38 (35–39)^3	61 (37–69)^5	36† (28–43)^3
	B	37 (32–40)^3	57 (36–69)^5	35† (28–39)^3

* See footnote to Table 6.

† See footnote to Table 3.

(free plus combined) to 5% (4–5% in three experiments). The corresponding average values for the rabbit (dose 208 μmoles/100 g.) were 45, 12 and 22% (Bray *et al.* 1957). Anorexia prevented satisfactory determination of glucosiduronic acid. Thus only 20% of the dose was accounted for as these three metabolites in the guinea pig, compared with 79% in the rabbit, which excreted an additional 13% of the dose as glucosiduronic acid. It is unlikely that the guinea pig excreted a large amount of glucosiduronic acid since the naphthoresorcinol values for the urines were usually lower than those from the urine passed before the dose was given. Neither 3:4-dichloronitrobenzene nor 3:4-dichloroaniline was detected in the faeces. 3:4-Dichloroaniline was detected by paper chromatography in ether extracts A and B and 5-amino-2:3-dichlorophenol in extracts A, B, C and D of the urine. (For preparation of extracts A, B, C and D and methods of identification see Bray *et al.* 1956, 1957.) The presence of 2:3-dichloro-5-nitrophenol was also shown since the elution of one of the spots and reduction of the eluate gave 5-amino-2:3-dichlorophenol. No *o*-aminophenols were detected. *S*-(2-Chloro-4-nitrophenyl)-L-cysteine was not detected in a butanol extract of urine adjusted to pH 4·0. The substituted cysteine added to normal urine could be detected by this procedure. Examination of urines from guinea pigs which had received *S*-(2-chloro-4-nitrophenyl)-L-cysteine revealed the presence of at least five diazotizable compounds, none of which gave the indophenol reaction for *p*-aminophenols. Neither mercapturic acid nor the substituted cysteine was detected. Parke & Williams (1951*b*) reported that 24% of administered *S*-phenyl-L-cysteine was excreted unchanged by the rabbit.

Qualitative examination of urines

Butylmercapturic acid was readily detected on paper chromatograms of the urine excreted after the administration of bromobutane to the rabbit and rat, but only traces were found in the urine of a similarly treated guinea pig. No *S*-butylcysteine was detected on paper chromatograms from any of the urines by the reagent of Knight & Young (1958), but traces were detected by ninhydrin in the urine of rabbits and guinea pigs but not in that of rats. The unidentified metabolite (see Table 1) was detected in urines of the three species.

Butylmercapturic acid and traces of *S*-butylcysteine were detected by paper chromatography

Table 5. *Acetylation of amino compounds by tissue slices*

The basic medium, B, consisted of 10 ml. of Ringer phosphate buffer, pH 7·4, containing 2·5 mg. of substrate and 20 mg. of glucose. Medium A was the same except that 27·2 mg. of sodium acetate ($CH_3 \cdot CO_2Na, 3H_2O$) was added. After addition of tissue slices the system was incubated with continuous shaking for 4 hr. at 37° with O_2 as gas phase. Duplicate 1 ml. samples were withdrawn for analysis at 0 and 4 hr. The results shown are for liver slices. Similar experiments with 2 g. of kidney slices gave negative results except that a trace of acetylation of *S*-(*p*-chlorobenzyl)-L-cysteine was detected with rat kidney. Values are the means of duplicate determinations in close agreement. A values immediately below B values were obtained from experiments in which the same sample of liver was used.

Digest	Wt. of slices (g.)	Percentage of substrate acetylated		
		Rabbit	Rat	Guinea pig
Substrate: *p*-aminobenzene sulphonamide				
B	1	17, 14, 14	19, 12, 9, 8	7
A	1	20	26	13
B	2	39	38	19
A	2	44, 76, 47	39	21, 26
Substrate: D-glucosamine				
B	1	< 1, < 1	8, 6, 8	0, 0
A	1	< 1, < 1	14, 9, 13	0, 0
B	2	10, 7, 5	25	< 1, < 1
A	2	10, 9, 5	30	< 1, < 1
Substrate: *S*-(*p*-chlorobenzyl)-L-cysteine				
B	2	9, 10	9, 8	< 1, < 1
A	2	12, 10	9, 8	< 1, < 1
Substrate: *S*-butyl-L-cysteine				
B	2	11*	5	11, 5*
A	2	17*	6	13, 9*

* Very little of this appears to be true mercapturic acid; see text for results of paper chromatography of end products of similar experiments.

in the urines of rabbits and rats to which *S*-butylcysteine had been given but they were not detected in the urine of guinea pigs similarly treated. The unidentified metabolite was detected in the urine of all three species.

Neither the unchanged compound nor the corresponding mercapturic acid was detected on paper chromatograms of the urine of guinea pigs to which *S*(2:3:5:6-tetrachlorophenyl)-L-cysteine had been administered. An unidentified metabolite (see Table 1) was detected. The mercapturic acid was not detected in the ethereal extract of acidified urine. On acidification of alkali-hydrolysed urine obtained from three guinea pigs which had each received 75 mg. of *S*(2:3:5:6-tetrachlorophenyl)-L-cysteine a precipitate formed. This was separated and recrystallized from aqueous acetone, when 10 mg. of impure crystals, m.p. 75°, were obtained which gave a positive Folin–Ciocalteu test and a spot which was indistinguishable from 2:3:5:6-tetrachlorothiophenol on paper chromatograms. Recrystallization from aqueous acetone yielded crystals, m.p. 130–134°, admixture with which did not depress the m.p. of the presumed disulphide (see Bray *et al.* 1953).

DISCUSSION

Tables 3 and 6 show that the guinea pig forms mercapturic acids less readily than does the rabbit or the rat. By the method of determination used 1-bromobutane and *S*-butyl-L-cysteine appeared to yield about 19 and 31 % respectively of the dose as mercapturic acid but only traces of butylmercapturic acid could be detected in the urines. Grenby & Young (1959) detected propylmercapturic acid in the urine of guinea pigs dosed with 1-bromopropane. The results in Table 4 suggest that insignificant amounts of *S*-butylcysteine were excreted after the administration of either 1-bromobutane or *S*-butylcysteine; only traces of *S*-butylcysteine were detected on paper chromatograms. The analytical results could be accounted for by the presence in the urines of a compound (or compounds) which yields 1-mercaptobutane on hydrolysis with sodium hydroxide but which is neither *S*-butyl-L-cysteine nor butylmercapturic acid. The guinea pig also appeared to excrete a high proportion of the dose of *S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine as mercapturic acid but again no mercapturic acid was isolated from the urine, although *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine has been easily isolated on many occasions from rabbit urine. Neither the mercapturic acid nor the *S*-tetrachlorophenylcysteine could be detected by paper chromatography of guinea-pig urine. As *S*-(2:3:5:6-tetrachlorophenyl)cysteine added to urine is completely removed by the zinc hydroxide used in the iodometric method it is unlikely that the excretion of unchanged material accounts for the high iodine uptake of the hydrolysed guinea-pig urine. The tetrachlorothiophenol obtained on alkaline hydrolysis of the urine is presumably derived from an unidentified metabolite which is not formed to any significant extent when 2:3:5:6-tetrachloronitrobenzene is administered. By contrast, an unidentified sulphur-containing metabolite is excreted when either bromobutane or *S*-butylcysteine is given to guinea pigs. A possible metabolite which would account for the observed results would be the corresponding deaminated cysteine, e.g. *S*-butyl-3-mercaptopyruvic acid and *S*-(2:3:5:6-tetrachlorophenyl)-3-mercaptopyruvic acid. Keto acids of this type

Table 6. *Excretion of mercapturic acid by the rabbit, rat and guinea pig after administration of precursors*

Precursor administered	Mean percentage of dose excreted as mercapturic acid		
	Rabbit	Rat	Guinea pig
Naphthalene	4[1]	12[2]	0·6[3]
Chlorobenzene	20[4]	18[5]	1[6]
Benzyl chloride	49[7]	27[5]	4[6]
p-Chlorobenzyl chloride	23[7]	37[5]	5[6]
1-Bromobutane	45[5]	44[5]	19[6]*
3:4-Dichloronitrobenzene	45[8]	19[5]	3[6]
2:3:4:6-Tetrachloronitrobenzene	37[9]	32[5]	2[6]
2:3:5:6-Tetrachloronitrobenzene†	14[10]	11[5]	0[6]

(1) By isolation, Bourne & Young (1934).
(2) By isolation, Young (1947).
(3) By isolation, Corner & Young (1954).
(4) Spencer & Williams (1950).
(5) Barnes, James & Wood (1959).
(6) Bray, Franklin & James (1959).
(7) Bray, James & Thorpe (1958).
(8) Bray, James & Thorpe (1957).
(9) Betts, James & Thorpe (1955).
(10) Bray, Hybs, James & Thorpe (1953).

* The true value is probably very small since no butylmercapturic acid could be detected in the urines by paper chromatography (see text).

† Only 34 % of a dose of this compound is absorbed by the rabbit, 76 % by the rat and 82 % by the guinea pig.

have been identified as metabolites of *S*-benzylcysteine and *S*-benzylhomocysteine by Shen & Lewis (1946). Roberts & Warwick (1958), however, concluded that ethylmercaptopyruvic acid was not a metabolite of ethyl methanesulphonate.

It is unlikely that absorption difficulties could account for the failure of the guinea pig to excrete mercapturic acid, since even with 2:3:5:6-tetrachloronitrobenzene about 82% of the dose appears to be absorbed. The corresponding values for the rabbit and the rat are 34% (Bray *et al.* 1953) and 76% respectively. The results of the experiments with tissue slices (Table 5) also indicate that D-glucosamine and *S*-(*p*-chlorobenzyl)-L-cysteine were acetylated to a significant extent by liver slices from the rabbit and rat but not by those from the guinea pig. *p*-Aminobenzene sulphonamide was, and *S*-butylcysteine appeared to be, readily acetylated by liver slices of all three species although it is doubtful whether more than a trace of mercapturic acid was actually formed from *S*-butylcysteine by guinea-pig or rabbit liver. The results obtained from liver slices therefore are in accord with the results obtained *in vivo* except for the failure to detect significant formation of butylmercapturic acid in rabbit-liver slices. The reason for this finding has not been discovered.

The guinea pig provides another example of a species difference in acetylation. It is well known (e.g. Marshall, Cutting & Emerson, 1937; Krebs, Sykes & Bartley, 1947) that the dog excretes only a small proportion of administered aminoaryl sulphonamide as acetyl derivatives. Although the guinea pig readily acetylates *p*-aminobenzene sulphonamide it appears to have difficulty in acetylating the aliphatic amines examined. This may be due to the specificity of the enzyme responsible for transferring the acetyl group from acetyl-coenzyme A to the amine. Examples of such specificity have been given by Tabor, Mehler & Stadtman (1953), who obtained a pigeon-liver preparation which would acetylate *p*-aminobenzoic acid, *m*-nitroaniline, *o*-phenylenediamine, *o*- and *m*-toluidine, *o*- and *p*-bromoaniline and *o*- and *p*-anisidine but not *o*-aminobenzoic acid, *p*-nitromethylaniline or kynurenine. Davidson *et al.* (1957) obtained a preparation from *Neurospora crassa* which acetylated glucosamine 6-phosphate, but not glucosamine, galactosamine, *p*-nitroaniline or amino acids. The results of Krebs *et al.* (1947) suggest that the apparent inability of the dog to acetylate aromatic amines may be due to an active deacetylation process which occurs simultaneously. This deacetylating system is inactive in the intact rabbit. Experiments are in progress to test whether the apparent failure of the guinea pig to acetylate aliphatic amino compounds can be attributed to a deacetylation process.

None of the results reported in this and the two earlier papers (Barnes *et al.* 1959; Bray *et al.* 1959) provides an objection to the hypothesis that for the compounds examined mercapturic acids are formed by a three-stage process consisting of: (1) the conjugation of the precursor with glutathione, (2) the hydrolysis of the glutathione conjugate by glutathionase to an *S*-substituted cysteine, glycine and glutamic acid and (3) the acetylation of the *S*-substituted cysteine to mercapturic acid. Reactions 2 and 3 can occur in the liver of the rabbit and rat, but direct proof that reaction 1 can occur is desirable before the hypothesis can be regarded as established. The comparative failure of the guinea pig to excrete mercapturic acids is probably not due to a failure of reactions 1 or 2 but rather to a defect in reaction 3, since even when *S*-substituted cysteines are administered the guinea pig excretes relatively small amounts of the dose as mercapturic acid.

SUMMARY

1. The *N*-acetylation of some *S*-substituted cysteines by the rabbit, rat and guinea pig *in vivo* and their tissues *in vitro* has been studied.

2. Unlike the rabbit and the rat, the guinea pig did not excrete as mercapturic acid a large proportion of a dose of any of the six *S*-substituted cysteines examined.

3. The extent of acetylation of *S*-(*p*-chlorobenzyl)cysteine and *S*-butylcysteine *in vitro* with liver slices was comparable with that obtained *in vivo*. *p*-Aminobenzene sulphonamide was readily acetylated by the guinea pig and by slices of guinea-pig liver.

4. The failure of the guinea pig to excrete considerable amounts of mercapturic acid from precursors appears to be due to difficulty in *N*-acetylation of *S*-substituted cysteines.

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Kinetic Studies on Rabbit-Muscle Myokinase

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Myokinase (adenylate kinase) catalyses the reaction:

2 Adenosine diphosphate ⇌ Adenosine triphosphate + adenosine monophosphate (1)

Highly purified preparations of this enzyme have been obtained from rabbit muscle by Noda & Kuby (1956, 1957*a*, *b*) and Callaghan (1956, 1957*a*, *b*), and the preparation of the last-named author, which appears to contain a single protein component, has been used for the present studies of the mechanism of myokinase action. Several possible mechanisms by which myokinase might catalyse transphosphorylation among adenosine nucleotides were considered by Whittam, Bartley & Weber (1955). The enzyme may accept the terminal phosphate of adenosine triphosphate (ATP) or adenosine diphosphate (ADP), thus forming an intermediate phosphorylated protein by the reactions:

Enzyme + ATP ⇌ Enzyme–P + ADP (2)

Enzyme–P + adenosine monophosphate (AMP) ⇌ Enzyme + ADP (2*a*)

or may act by forming an enzyme–nucleotide complex according to the following scheme:

Enzyme + APPP + AP ⇌ Enzyme–APP⋮PPA ⇌ Enzyme + APP + APP (3)

A further possibility is expressed by the reactions:

Enzyme + ATP ⇌ Enzyme–ATP
Enzyme–ATP + AMP ⇌ Enzyme–ADP + ADP (4*a*)

Enzyme + AMP ⇌ Enzyme–AMP
Enzyme–AMP + ATP ⇌ Enzyme–ADP + ADP (4*b*)

By use of the purified enzyme, the hypothetical phosphorylated enzyme intermediate was tested for directly by incubation with ATP of comparable molar concentration in the region of the Michaelis constant (K_m) value for the substrate; in most experiments of this kind, the ATP was labelled with ^{32}P in the β- and γ-phosphate groups. Kinetic studies were also undertaken to determine the affinity of the enzyme for its substrates, and a special study of the reverse reaction was made to

determine whether the enzyme contained sites to activate two molecules of substrate simultaneously or a single site (as expressed by equations 3 and 4 respectively). The preparation was also tested for other transphosphorylating enzymes.

EXPERIMENTAL

Adenosine triphosphate. This was obtained as the crystalline disodium salt from the Sigma Chemical Co., St Louis, Mo., U.S.A. Chromatographic analysis of this material by the method of Krebs & Hems (1953) indicated that 94% of the total phosphate was present in the form of ATP, 4–5% as ADP and approximately 2% as inorganic orthophosphate (PI). These figures are discussed in greater detail below.

Adenosine diphosphate. This was obtained as the crystalline disodium salt from the Sigma Chemical Co. and was found on analysis to contain less than 2% of AMP.

Adenosine monophosphate. This was obtained from Roche Products Ltd., Welwyn Garden City, Herts. Solutions of AMP were brought to pH 7·5 before use.

Inosine and uridine phosphates. The authors gratefully acknowledge the gift of small amounts of these compounds by Dr N. L. Blumsom; all were 5′-phosphate esters. The inosine 5′-phosphate preparation contained much inosine 5′-diphosphate (see below) but was not purified further.

Cytidylic acid and guanylic acid. These were obtained as the 3′-phosphate esters from L. Light and Co. Ltd., Slough, Bucks.

Adenosine 5′-monosulphate. A small amount of this substance was the generous gift of Professor Kunio Yagi.

$KH_2{}^{32}PO_4$. This was supplied by The Radiochemical Centre, Amersham, Bucks.

Chromatographically pure adenosine triphosphate. ATP (Sigma Chemical Co.) was rechromatographed with the intention of removing traces of ADP. The dissolved substance was applied as a streak to Whatman no. 3 paper and the procedure of Krebs & Hems (1953) was followed. The ATP on the paper was localized by illumination with the 2537 Å Hg line. This region was cut out, washed with ether to remove traces of solvents and eluted by downward chromatography with water. Chromatographic analysis of this product is described below.

[^{32}P]*Adenosine triphosphate.* This compound, containing equal specific activity in the β- and γ-phosphate groups (AT$\beta\gamma^{32}$P) was prepared by a procedure (similar to that of Hems & Bartley, 1953) of Dr M. L. Bobb (personal communication). The gift of an initial sample of AT$\beta\gamma^{32}$P from Dr Bobb is gratefully acknowledged. A low-phosphorus yeast extract was prepared according to Eggleston (1954). A portion (2 ml.) of the extract was shaken aerobically at room temperature with 0·1 ml. of 0·02 M-ATP (adjusted to pH 6·8) and 2 ml. (5 mc) of $KH_2{}^{32}PO_4$ solution (pH 7·0) for 4 hr. The mixture was transferred to a conical centrifuge tube and deproteinized with 0·5 ml. of 50% (w/v) trichloroacetic acid soln. The supernatant obtained on centrifuging was cooled to 0° and applied in a streak to Whatman no. 3 paper, the AT$\beta\gamma^{32}$P being separated by chromatography as described above. A representative preparation of 0·25 μ-mole of AT$\beta\gamma^{32}$P gave a total count of $2{\cdot}9 \times 10^6$/min. in solution. Of the total counts, ATP contained 74%, ADP 5%, AMP 0·2% and PI 20%. The specific activity of the β- or γ-phosphate groups of ATP was $2{\cdot}1 \times 10^5$ counts/min./μg. of phosphorus, the α-phosphorus atom being negligibly labelled. The preparation was stored on a Whatman no. 3 paper strip at $-15°$, and small quantities were eluted with ATP solution for use in experiments.

Myokinase. This was prepared by the method of Callaghan (1957*b*) and was dialysed in collodion tubes coated with cytochrome *c* (Callaghan, 1957*b*).

Assay methods

Myokinase. For the routine assay of myokinase preparations the method of Oliver (1955) was used. In kinetic studies, the initial velocity of myokinase activity was measured by chromatographic assay as previously described (Callaghan, 1957*b*). It was found that the chromatographic procedure of Krebs & Hems (1953) was also suitable for the separation of uridine di- and mono-phosphate from the adenosine nucleotides, the former compounds having similar R_F values to inosine di- and mono-phosphate respectively. Cytidylic acid had an almost identical R_F value with that of AMP.

Radioactivity measurements. These were made in an M6 liquid counter (Veall, 1948), which was periodically cleaned with a dilute solution of detergent (Teepol; Shell) to remove adsorbed protein and traces of radioactive material. Areas on chromatograms were cut as circles from the paper and counted in an end-window counter. Corrections were made for dead-time and background counts. No correction was made for isotope decay, as standard solutions of AT$\beta\gamma^{32}$P were counted with all specimens. Spectrophotometric measurements were made with an SP. 500 Unicam spectrophotometer.

Estimation of nucleotides in solutions of protein. In some experiments it was necessary to estimate the sum of the concentrations of ATP, ADP and AMP in solution by spectrophotometry (these compounds have identical absorption spectra in the ultraviolet region). It was also necessary to correct for the light absorption due to myokinase protein present. As will be shown below, the characteristic absorption of ultraviolet light by the enzyme and the nucleotides in solution is purely additive. The following equations were therefore applicable.

Let the ratio of light extinction by myokinase protein at the wavelengths

$$260 \text{ m}\mu \text{ and } 280 \text{ m}\mu \text{ be } \frac{P_{280}}{P_{260}} = \pi.$$

Similarly, for ATP, ADP and AMP, the ratio of extinction at the same wavelengths

$$\frac{N_{280}}{N_{260}} = \nu.$$

(At the low concentrations used for measurement, where the total extinction was 0·1–0·2, the average values for π and ν were 1·28 and 0·178 respectively.)

The total absorption of light at 260 mμ, $E_{260} = P_{260} + N_{260}$.

Similarly, the total absorption at 280 mμ,

$$E_{280} = P_{280} + N_{280}.$$

Therefore
$$E_{280} = \pi P_{260} + \nu N_{260} \quad (5)$$

and
$$P_{280} = E_{260} - N_{260}. \quad (6)$$

Combining equations (5) and (6),

$$E_{260} = \pi E_{260} + (\nu - \pi)\, N_{260}.$$

Therefore

$$N_{260} = \frac{E_{260} - \pi E_{260}}{(\nu - \pi)}. \qquad (7)$$

From equation (7) it is possible to obtain the extinction at 260 mμ due to nucleotide alone with reasonable precision, since $(\nu - \pi)$ is approximately unity.

RESULTS

Tests for a phosphorylated enzyme intermediate

Direct method. To determine whether myokinase formed a phosphorylated intermediate, according to equation (2), ATP and myokinase were incubated together in comparable molar concentrations which were of the order of the K_m concentration of ATP. The results of this experiment are given in Table 1, and show that there was no alteration in the proportions of ATP and ADP; this indicates that myokinase did not form a phosphorylated intermediate, otherwise the enzyme (0·9 μmole) would have been approximately half-saturated with phosphate, with the concomitant dephosphorylation of about 26 % of the ATP present to form ADP. Table 1 also shows that the chromatographically separated specimen of ATP had the same proportions of ATP, ADP and PI as the original preparation from which it was made. The conclusion is drawn that some hydrolysis of ATP (about 5 %) occurs during chromatography in the first solvent of Krebs and Hems (*iso*propyl ether–formic acid), and that the Sigma Chemical Co. preparation of ATP contains a higher percentage of this compound than this analysis would indicate, being possibly 98–99 % pure as claimed by the manufacturers.

Table 1. *Test for phosphorylated enzyme intermediate*

In a total volume of 4 ml. the following constituents were incubated at 20° and pH 7·4: 18·9 mg. of pure myokinase (0·865–0·9 μmole); 0·01 ml. of M-$MgCl_2$; 20 mg. of cysteine hydrochloride (neutralized to pH 7·4); 1·7 μmoles of chromatographed ATP (final concentration 0·426 mM; K_m of ATP = 0·3 mM, see below). After 1 min. 1 ml. of 30 % (w/v) trichloroacetic acid was added, and the mixture was cooled to 0° and centrifuged. A portion (172 μl.) of the supernatant was applied in a streak on Whatman no. 1 paper and chromatographed according to Krebs & Hems (1953). 93 % of the total phosphates added to the above reaction mixture was recovered from the trichloroacetic acid supernatant.

	Percentage of total phosphorus as phosphate in chromatographic spots		
Area	ATP (Sigma Chemical Co.)	Chromatographically separated ATP	Trichloroacetic acid supernatant from enzyme
ATP	94·5	93·5	95·0
ADP	4·5	4·5	4·0
PI	1·0	2·0	1·0

Experiments with radioactive adenosine triphosphate. The possible phosphorylation of myokinase by ATP was also tested for by incubation of the enzyme with AT$\beta\gamma^{32}$P. Attempts were made to separate the enzyme–$^{32}PO_4$ complex from the added nucleotide by means of prolonged dialysis and zone electrophoresis.

It was expected that, if such a complex were formed, its partial or total separation from the radioactive nucleotide would be reflected in a high specific radioactivity of the enzyme, for which any residual nucleotide could not be responsible, i.e. the ratio $\frac{\text{counts/unit time}}{\text{moles of adenosine nucleotide}}$ would be greater than that of the added AT$\beta\gamma^{32}$P.

In a representative experiment, shown in Table 2, myokinase and AT$\beta\gamma^{32}$P were dialysed in the presence of 0·1 M-KCl, the latter substance being employed to prevent a Donnan distribution of nucleotide on either side of the membrane. At this concentration KCl does not inhibit myokinase, whereas M-KCl almost entirely inhibits the enzyme (Bowen & Kerwin, 1954). It can be seen that the enzyme was not completely separated from the added nucleotide, since the extinction at 260 mμ was always greater than at 280 mμ. The counts/N_{260} ratio for the inner, enzyme-containing solution remained similar to that of the AT$\beta\gamma^{32}$P standard, within the limits of experimental error due to pipetting, counting and spectrophotometric measurement, until the third dialysis. After this, the ratio fell below that of the standard so that the nucleotide in the enzyme solution must have consisted largely of ADP, formed by the gradual hydrolysis of ATP.

Table 2 also shows that the addition of *p*-chloromercuribenzoate caused a more rapid loss of radioactivity from the enzyme solution during dialysis.

It was observed that at the termination of the above-mentioned experiment (which continued for 9 days), a small protein precipitate had appeared in the membrane tubes, and only 20–50 % of the expected enzyme activity could be recovered in tubes 1–3. In the tubes containing *p*-chloromercuribenzoate the enzyme was entirely inactive, but 60–75 % of the expected activity could be assayed after activation with cysteine (Callaghan, 1957*b*).

After dialysis, the solutions were analysed chromatographically in the presence of 'carrier' amounts of ATP, ADP, AMP and PI, and the areas counted. Table 3 shows the results of this analysis. The areas on the starting line of the chromatogram

Table 2. *Dialysis of myokinase after addition of adenosine [^{32}P]triphosphate*

Dialysis was carried out in collodion tubes coated with cytochrome *c*. Constituents present in tubes 1–3 at zero time were: 5·15 mg. of pure myokinase; 0·385 mM-ATP (containing AT$\beta\gamma^{32}$P); 15 mM-tris buffer, pH 7·5; 2 mM-$MgCl_2$; 0·1 M-KCl; 10 mg. of neutralized cysteine hydrochloride. Total volume, 4 ml. Tubes *p*CMB1 and *p*CMB2 contained 0·84 mM-*p*-chloromercuribenzoate initially, in addition to the above constituents, but contained no cysteine. Each tube was dialysed separately at 5–8° against 8 ml. of 0·1 M-KCl containing 15 mM-tris buffer, pH 7·5, and subsequently against two lots of 200 ml. of buffered KCl solution. Preliminary experiments with the coated collodion tubes showed that dialysis equilibrium for ATP was reached in 48–55 hr. The value of N_{260} was calculated from equation (9) and the counts/N_{260} ratio was estimated for standard solutions of AT$\beta\gamma^{32}$P, which were counted with each set of samples taken.

(*a*) *First dialysis (against 8 ml. of buffered* KCl *soln.*). Samples (0·1 ml.), taken from inside and outside solutions, were diluted to 2 ml. for spectrophotometry and then to 10 ml. for counting at the stated time intervals.

	20 hr.				48 hr.				68 hr.			
Tube	E_{260}	E_{280}	Counts/ 5 min.	$10^{-4}\times$ (Counts/ N_{260})	E_{260}	E_{280}	Counts/ 5 min.	$10^{-4}\times$ (Counts/ N_{260})	E_{260}	E_{280}	Counts/ 5 min.	$10^{-4}\times$ (Counts/ N_{260})
1	0·150	0·056	1870	1·51	0·104	0·038	1551	1·78	0·103	0·035	1462	1·76
2	0·135	0·055	1840	1·72	0·109	0·044	1465	1·68	0·106	0·038	1475	1·65
3	0·127	0·058	1702	1·79	0·110	0·048	1485	1·76	0·109	0·043	1520	1·72
*p*CMB1	0·165	0·058	1957	—	0·134	0·045	1523	—	0·125	0·040	1500	—
*p*CMB2	0·193	0·080	1916	—	0·160	0·063	1061	—	0·155	0·060	1578	—
AT^{32}P standard	0·161	—	3023	1·87	0·161	—	2779	1·73	0·161	—	2774	1·72

(*b*) *Second dialysis*. The tubes were rapidly rinsed on the outside with water, and each tube was dialysed against 200 ml. of buffered KCl solution. After 67 hr. samples (0·5 ml.) were diluted to 2·5 ml. for spectrophotometry and then to 10 ml. for counting.

(*c*) *Third dialysis*. The tubes were dialysed against 200 ml. of fresh buffered KCl solution. After 48 hr. samples (1·0 ml.) were taken, diluted to 2·5 ml. for spectrophotometry and then to 10 ml. for counting.

Tube	E_{260}	E_{280}	Counts/ 20 min.	$10^{-4}\times$ (Counts/ N_{260})	E_{260}	E_{280}	Counts/ 20 min.	$10^{-4}\times$ (Counts/ N_{260})
1	0·059	0·045	1414	5·1	0·196	0·161	2928	3·53
2	0·095	0·081	2167	5·9	0·205	0·173	2933	3·58
3	0·083	0·070	1754	5·35	0·280	0·229	4260	3·6
*p*CMB1	0·045	0·030	570	—	0·073	0·063	1019	—
*p*CMB2	0·108	0·088	634	—	0·154	0·142	764	—
AT^{32}P standard	0·129	—	7146	5·6	0·129	—	6361	4·92

contained the myokinase protein, as well as a small amount of material, presumably nucleotide, which gave a dark spot when illuminated by the 2537 Å Hg line.

Since prolonged dialysis of myokinase led to a gradual inactivation of the enzyme, more rapid separation of the enzyme from added AT$\beta\gamma^{32}$P was undertaken, the zone-electrophoresis apparatus of Porath (1954) being used. Figs. 1 and 2 show representative experiments in which partial and complete separations were achieved. In Fig. 1 it is shown that the counts/N_{260} ratio fell sharply from the standard value of the nucleotide fractions to a low value in the enzyme-containing fractions; after complete separation (Fig. 2) the protein contained some firmly bound nucleotide, but gave no significant count above the background count. From these results it is inferred that the nucleotide associated with the enzyme was AMP (since this compound would be negligibly labelled with ^{32}P). This trace of AMP presumably arose from the slow hydrolysis of ATP, coupled with enzyme activity. In the experiment given in Fig. 2, the AT$\beta\gamma^{32}$P migrated completely out of the electrophoresis column into the tank of buffer, where its presence was detected by the radioactivity of the buffer.

Absorption spectra of myokinase and adenosine triphosphate in solution. In the above-mentioned experiment, it was observed that nucleotide appeared in the enzyme fractions after removal of ATP by electrophoresis. The absorption spectra of these solutions were compared with those of mixtures of myokinase and nucleotides at known concentrations.

Fig. 3 shows the spectrum of a representative myokinase preparation used for these studies; a similar spectrum has been published by Noda & Kuby (1957*b*) for crystalline myokinase. A well-defined trough is present in the spectrum at 250 mμ, the ratio E_{279}/E_{250} for our preparation being 5·6, whereas that for the preparation of Noda & Kuby is 2·3, as measured from their published spectrum. The presence of traces of nucleotide fills the trough at 250 mμ and, at higher concentrations, shifts the maximum from 279 mμ to the region of 265 mμ. The resulting spectrum, however, is purely the sum of the spectra of the two components. The spectrum of a myokinase fraction (fraction 19 in Fig. 2) which contained traces of bound nucleotide, presumably AMP, after electrophoretic removal of ATP showed that the

Table 3. *Chromatographic separation of radioactive material in dialysed enzyme nucleotide solution*

Specimens (0·2 ml.) from each tube from the dialysis experiment (Table 2) were spotted on to Whatman no. 1 paper. On each spot 5 μl. of 0·02 M-ATP, -ADP, -AMP and -PI was superimposed to act as carriers for traces of radioactive phosphates. After chromatography (Krebs & Hems, 1953) the areas were identified by viewing in ultraviolet light, and PI was located by the molybdate spray and H_2S (Hanes & Isherwood, 1949). The total areas were cut as circles for counting; 20 min. counts of each area are given. *p*CMB1, *p*CMB2: tubes contained *p*-chloromercuribenzoate initially (see Table 2).

Specimen	PI	ATP	ADP	AMP	Starting spot (protein)
1	68	11	0	0	461
2	18	23	0	0	301
3	134	31	32	0	666
*p*CMB1	7	5	0	0	13
*p*CMB2	41	60	—	—	23

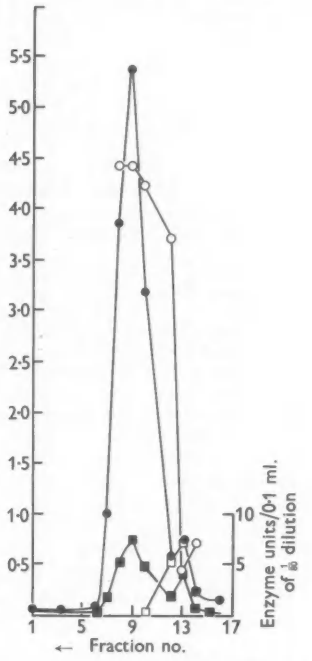

Fig. 1. Partial separation of myokinase from AT$\beta\gamma^{32}$P by zone electrophoresis. The column of the electrophoresis apparatus contained borate–phosphate buffer, pH 8·4, μ 0·05. The following mixture was introduced as a zone into the column in a total volume of 6·01 ml.: 15 mg. of myokinase (previously dialysed against buffer); 1 ml. of 10 mM-AT$\beta\gamma^{32}$P; 15 mg. of cysteine hydrochloride neutralized to pH 8·4; 0·01 ml. of M-$MgCl_2$. After electrophoresis for 9 hr. at 8°, with a current of 22 mA (140 v, D.C.), 10 ml. fractions were collected from the column. ○, 10^{-4} × (Counts/N_{260} ratio); ●, extinction at 260 mμ; ■, extinction at 280 mμ; □, enzyme activity. The arrow indicates the direction of migration towards the cathode.

ratio E_{279}/E_{250} was altered to 1·0, and an absorption maximum was present at 265 mμ.

Previous experience has shown that the enzyme binds traces of nucleotides very tenaciously during the process of purification. It is not suggested that this type of binding is necessarily related to the formation of enzyme–substrate complexes.

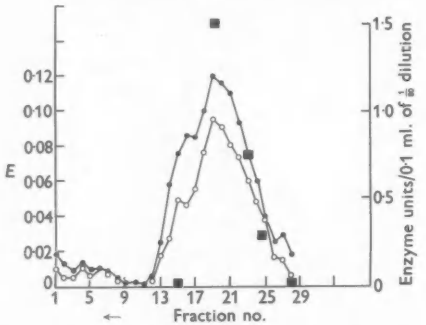

Fig. 2. Total separation of myokinase–nucleotide complex from AT$\beta\gamma^{32}$P by zone electrophoresis. The column contained borate–phosphate buffer, pH 8·4, μ 0·05. The following mixture was introduced into the column as a zone in a total volume of 5·01 ml.: 10 mg. of myokinase (previously dialysed against buffer); 1 ml. of 10 mM-AT$\beta\gamma^{32}$P; 15 mg. of cysteine hydrochloride neutralized to pH 8·4; 0·01 ml. of M-$MgCl_2$. After electrophoresis for 27·75 hr. at 8°, with a current of 21 mA (approx. 140 v, D.C.), 7 ml. fractions were collected from the column. ●, Extinction at 260 mμ; ○, extinction at 280 mμ; ■, enzyme activity. The arrow indicates the direction of migration towards the cathode.

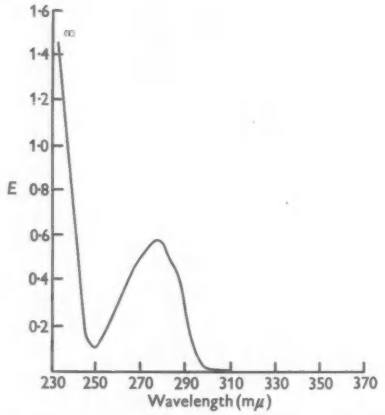

Fig. 3. Absorption spectrum of myokinase (0·51 mg./ml.) in 0·01 M-phosphate buffer, pH 7·0 (see text).

Kinetic studies

Inhibitor constants of adenosine tri- and mono-phosphate and p-*chloromercuribenzoate.* A comparison of the initial velocities of the forward and

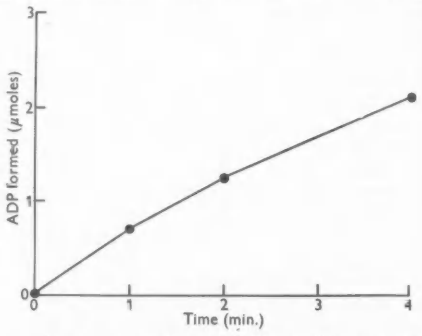

Fig. 4. Progress curve of reaction ATP + AMP ⟶ 2ADP at 30°. The constituents present were: 6 mM-ATP; 6 mM-AMP; 30 mM-tris buffer, pH 7·5; 2 mM-$MgCl_2$; 1 μg. of myokinase. Volume at zero time, 1·6 ml. Samples were removed at times indicated and treated with 0·1 vol. of 30% (w/v) trichloroacetic acid.

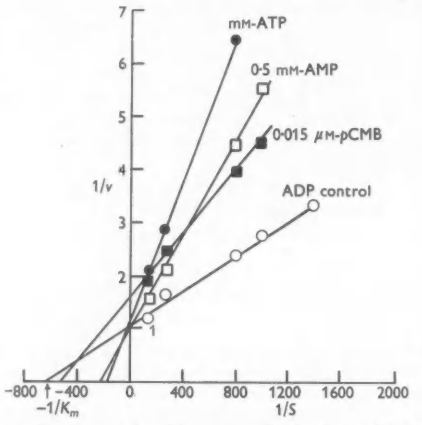

Fig. 5. Representative Lineweaver–Burk plots of initial velocity of myokinase reactions at 30° by the technique of Callaghan (1957*b*), with varied concentration of ADP, and inhibition by mM-ATP and 0·5 mM-AMP (competitive) and 0·015 μM-*p*-chloromercuribenzoate (*p*CMB) (non-competitive). At zero time, in a total volume of 1·6 ml., the constituents present were: ADP (as indicated in the figure); 0·03 M-tris buffer, pH 7·5; 2 mM-$MgCl_2$; 0·5 μg. of myokinase. Inhibitors were present in the reaction mixtures as indicated. Initial velocity in μmoles of ADP dismuted was calculated for 1 min. of reaction (v), at each concentration of ADP (S). Intercept for ADP control plot at $-1/K_m$ gives a K_m value of 1·58 mM.

reverse reactions catalysed by myokinase showed that whereas the velocity of the forward reaction (2ADP ⟶ ATP + AMP) decays rapidly with time (Callaghan, 1957*b*), the velocity of the reverse reaction is more constant (Fig. 4) and proceeds at 25–30 % of the initial rate of the forward reaction when the concentrations of ATP and AMP are separately equal to that of the ADP used in the forward reaction. These differences are believed to be due partly to the inhibitory effect of AMP (Colowick & Kalckar, 1943; Oliver, 1955); Fig. 5 shows that both AMP and ATP competitively inhibit the forward reaction, having enzyme–inhibitor dissociation constants (K_i) of 0·5 mM and 0·32 mM respectively, as calculated according to Massart (1950). Thus the formation of these substances by the forward reaction is followed by deceleration.

p-Chloromercuribenzoate inhibited myokinase non-competitively (Fig. 5). At a concentration of 8 mM-ADP, equimolar amounts of *p*-chloromercuribenzoate and myokinase (0·015 μM) showed 50 % of the control activity. Addition of cysteine (5 mg./ml.) entirely abolished this inhibition. The K_i value for *p*-chloromercuribenzoate was calculated from the formula $v/v_i = 1 + ([I]/Ki)$ (Massart, 1950) to be 0·015 μM.

It should be pointed out that myokinase has two SH groups (Callaghan, 1957*a*) and that this calculation, taken from a single measurement, is only satisfactory if it is assumed that (*a*) only one SH group need be blocked to produce inactivation and (*b*) the same group has a K_i very much smaller than the other SH group. If, for example, both groups had equal K_i values and both needed to be blocked for inactivation, total binding of the mercurial compound would result in the above case, giving 50 % inhibition, but with $K_i \to 0$. A more thorough study is required to clarify these points.

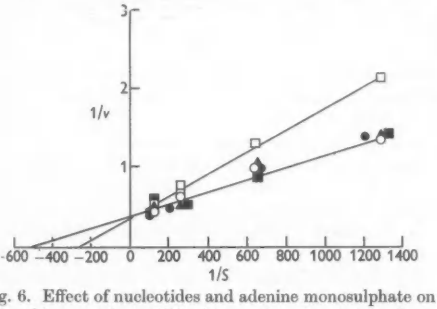

Fig. 6. Effect of nucleotides and adenine monosulphate on myokinase action at 30°. At zero time, in a total volume of 2·0 ml., the constituents present in the reaction mixtures were: 1 μg. of myokinase; 30 mM-tris buffer, pH 7·5; 2 mM-$MgCl_2$; ADP and nucleotides or adenine monosulphate as indicated. Reciprocal of velocity ($1/v$) in μmoles of ADP dismuted. Reciprocal of molar ADP concentration ($1/S$). The lower line shows the plot of the control reactions with ADP (points not shown). □, 0·0191 mM-Adenine monosulphate; ○, mM-adenosine; ▲, mM-ionosine triphosphate; ■, mM-cytidylic acid; ●, mM-uridine monophosphate.

Effect of other nucleotides and aromatic compounds on myokinase activity. At concentrations of 0·5 mM, inosine monophosphate, uridine monophosphate, cytidylic acid and guanylic acid gave no detectable inhibition, with concentrations of ADP ranging from 0·8 to 8·0 mM. Neither aniline nor benzoate (0·5 mM) showed any inhibition in the presence of 8 mM-ADP. At concentrations of mM no inhibition was produced by inosine monophosphate, uridine monophosphate, cytidylic acid or adenosine (Fig. 6), but adenosine monosulphate was shown to be a competitive inhibitor

(K_i = 0·0186 mM).

Cross-reactions between nucleotides catalysed by traces of contaminating enzymes in the myokinase preparation. Previous studies (Callaghan, 1957*b*) showed that the myokinase preparation was free of certain enzymes which might act on nucleotides. In Table 4 it is shown that the only reaction which took place was inosine triphosphate + AMP ⟶ inosine diphosphate + ADP, and the resulting ADP was dismuted by the excess of myokinase to give rise to ATP (reaction mixture 2). This indicated the presence of a trace of nucleoside monophosphate kinase activity (sufficient to cause the disappearance of approximately 0·025 μmole of inosine triphosphate/min.) in 16 μg. of protein. Since 1 μg. of myokinase transforms 0·66 μmole of ATP or AMP/min. the nucleoside monophosphate kinase activity would cause the removal of AMP (or inosine triphosphate) at a rate of 0·24 % of that of the myokinase, and no attempt was made to remove this trace of contaminant for the kinetic studies presented here. In reaction mixture 2, the presence of the contaminating enzyme caused a shift in the equilibrium of the myokinase reaction.

The equilibrium constant

$$K = \frac{[ATP]\,[AMP]}{[ADP]^2} = 0{\cdot}444$$

at pH 7·4 has been reported by Eggleston & Hems (1952) and Callaghan (1957*b*). In reaction mixture 2, K = 0·364. This effect has been noted by Gibson, Ayengar & Sanadi (1956). Nucleoside diphosphate kinase was absent from the preparation (mixture 1) and the myokinase equilibrium gave K = 0·42. In this mixture, the small amount of formed AMP was apparently not acted on by the monophosphate kinase during the incubation period. No reaction between ATP and either uridine di- or mono-phosphate was observed

Table 4. *Cross-reactions between adenosine nucleotides and other nucleotides catalysed by the myokinase preparation*

In a total volume of 0·6 ml. the constituents present in each reaction mixture at zero time were: 16 μg. of enzyme; 20 mM-tris buffer, pH 7·5; 1·5 mM-$MgCl_2$; nucleotides as indicated in the Table. Incubation was at 30° and samples (0·05 ml.) were withdrawn at the times indicated for treatment with 0·1 vol. of 30% (w/v) trichloroacetic acid. Nucleotides were separated chromatographically (Krebs & Hems, 1953) and estimated by phosphate analysis. All figures in the Table give the total amount of each nucleotide (in μmoles) calculated for the zero-time volume (0·6 ml.). ITP, Inosine triphosphate; IDP, inosine diphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate.

Reaction mixture ...	(1) ITP* + ADP			(2) ITP + AMP			(3) ATP + UMP		(4) ATP + UDP	
Time (min.)	0	2	30	0	2	30	0	30	0	30
Nucleotide										
ITP	0·58	0·56	0·56	0·55	0·50	0·13	—	—	—	—
IDP	0·38	0·37	0·38	0·32	0·36	0·76	—	—	—	—
ATP	—	0·70	0·68	—	—	0·14	1·27	1·31	1·40	1·50
ADP	2·4	1·10	1·05	—	0·08	0·27	0·06	0·08	0·11	0·13
AMP	0·1	0·71	0·69	2·32	2·24	1·90	—	—	—	—
UDP	—	—	—	—	—	—	—	—	0·38	0·39
UMP	—	—	—	—	—	—	2·10	2·17	—	—

* It should be noted that the ITP preparation contained about 40% of IDP.

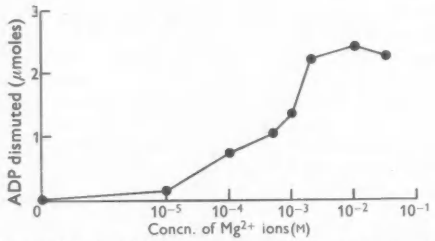

Fig. 7. Effect of Mg^{2+} ion concentration on the forward reaction at 30°. At zero time, the constituents present, in a total volume of 1·6 ml., were: 1 μg. of enzyme; 30 mM-tris buffer, pH 7·5; 80 mM-ADP. After 1 min. the reaction was stopped by addition of 0·1 vol. of 30% (w/v) trichloroacetic acid.

(reaction mixtures 3 and 4). The latter reaction is catalysed by the monophosphate kinase in yeast (Lieberman, Kornberg & Simms, 1954; Munch-Petersen, 1954), but this preparation (from rabbit muscle) was inactive towards uridine monophosphate.

Activation by magnesium. In the absence of Mg^{2+} ions no myokinase activity could be observed; thus the enzyme appeared to be free of bound magnesium. Fig. 7 shows that optimum activity was obtained at magnesium concentrations similar to that of the substrate concentrations. These results are in agreement with the finding of Kalckar (1943) that, at pH 7·5, enzyme activity is greatest in 0·01 M-$MgCl_2$, and appear to bear out the suggestion of Bowen & Kerwin (1954) that myokinase reacts with the magnesium complexes of adenosine phosphates. Kuby, Noda & Lardy (1954) have indicated that creatine phosphokinase also reacts with the magnesium complexes of ATP and ADP.

Inhibition by fluoride. The inhibitory effect of fluoride on myokinase activity in various tissues has been reported by Kotel'nikova (1950), Barkulis & Lehninger (1951), Leuhardt & Bruttin (1952) and Siekevitz & Potter (1953). The writers found that 0·02 M-sodium fluoride inhibits the forward reaction by 62% and the reverse reaction by 56% at pH 7·5 in the presence of 2 mM-$MgCl_2$. Kotel'nikova reported that 0·02 M-fluoride caused 43–48% inhibition (presumably of the forward reaction) of myokinase activity in extracts of rabbit muscle, heart and liver, and also noted that phosphate did not potentiate this inhibition. We have not studied the action of phosphate in fluoride inhibition.

Kinetics of the reverse reaction; theoretical introduction. In determining initial velocities for the reaction 2 ADP $\rightarrow$ ATP + AMP, it is necessary to take account of the rapid inhibition produced by the accumulated products. A simple best straight line connecting the experimental values for the velocity at different times is incorrect since the actual reaction course always shows a curvature concave towards the time axis. To obtain the initial velocity the tangent to the curve at time $\simeq 0$ must be obtained. The mirror method (Callaghan, 1957*b*) was used with this object.

In Fig. 5 it was shown that the reciprocal plot for the dismutation of ADP is linear, which suggested that the velocity of the forward reaction was a simple function of the ADP concentration. This implies that only one site is present on myokinase for the catalytic adsorption of ADP, since if two molecules of ADP were activated simultaneously, the following equation [see Dixon & Webb (1958)] will apply:

$$\frac{1}{v} = \frac{1}{V_{max.}}\left[1 + \frac{K_1}{A_0} + \frac{K_2}{B_0} + \frac{K_1 K_2}{A_0 B_0}\right],$$

where v is the velocity of reaction measured, $V_{max.}$ is the maximum velocity and K_1 and K_2 are the respective K_m values for substrates A and B, which are present at the concentrations A_0 and B_0. In the reaction 2 ADP $\longrightarrow$ ATP + AMP, $A_0 = B_0 = [ADP]$

$$\text{and} \quad \frac{1}{v} = \frac{1}{V_{max.}}\left[1+\frac{K_1+K_2}{[ADP]}+\frac{K_1K_2}{[ADP]^2}\right]. \qquad (8)$$

Thus on plotting $1/v$ against $1/[ADP]$, a curvature convex towards the $1/[ADP]$ axis would be observed. The departure from linearity would be due to the square term in [ADP].

In the range of concentrations which we could use no such deviation was observed, or at least the deviation was smaller than the experimental error. This did not rule out the possibility that the value for K_2 was so small that, even at the lowest concentration of substrate used, the second site was fully saturated with ADP.

If E is the vertical height of the usual reciprocal plot above infinite substrate concentration and if the linear law is followed with an experimental error smaller than the fractional departure from linearity, ΔE, down to a concentration $[ADP]_{min.}$, we have

$$\frac{\dfrac{K_1K_2}{[ADP]^2_{min.}}}{1+\dfrac{K_1+K_2}{[ADP]_{min.}}} < \frac{\Delta E}{E}.$$

$$\text{Thus} \quad \frac{K_1K_2}{[ADP]_{min.}} < \frac{\Delta E}{E}([ADP]_{min.}+K_1+K_2),$$

since $K_2 \ll K_1 \gg [ADP]_{min.}$

$$K_2 < \frac{\Delta E}{E}[ADP]_{min.}. \qquad (9)$$

Equation (9) gives the relation between the experimental error and the maximum possible value for K_2.

In the reciprocal plot for ADP given in Fig. 5, E was 2·35; the experimental error involved in the estimation of ADP by our methods is ±3%. At zero time 1·17 μmoles of ADP was present in the reaction mixture. After 1 min. of reaction, 0·3 μmole of ADP had been consumed ($1/v$ = 3·35). Thus 0·87 μmole of ADP remained for estimation; 6% of this is 0·05 μmole. This error could vary the estimation of the ADP content after 1 min. from, say, 0·87 to 0·82 μmole, thus varying $1/v$ from 3·35 (v = 0·3 μmole/min.) to 4·0 (v = 0·25 μmole/min.). The fractional departure, $\Delta E/E$, in the estimated height would be 0·65/2·35 = 0·276. From equation (9) $K_2 < 0{\cdot}276 \times 7{\cdot}3 \times 10^{-4}$ M-ADP. Therefore the maximum possible value for K_2 is 0·2 mM. With such a low value of K_2 it would be expected that ADP would be a powerful inhibitor of the reverse reaction, as it would compete for a site on the enzyme occupied by either ATP or AMP. This is implicit in the principle of microscopic reversibility, which holds that the intermediate complex formed at the surface of a catalyst in a reaction of the type A + B $\rightleftharpoons$ intermediate complex $\rightleftharpoons$ C + D is the same for the forward as for the reverse process.

Assuming the existence of two sites on the enzyme, the inhibitory effect of ADP at low concentrations (of the order of K_2) on the reaction ATP + AMP $\longrightarrow$ 2 ADP is given by equations (10) and (10a) below.

The following general considerations will also apply if myokinase possesses separate sites on the active centre for the two substrates ATP and AMP.

Let the enzyme have sites 1 and 2 for the specific adsorption of substrates 1 and 2 respectively with primary equilibrium constants $K_{1,1}$ and $K_{2,2}$. It is also possible that substrate 1 will be adsorbed at site 2, and substrate 2 at site 1, with constants $K_{1,2}$ and $K_{2,1}$ respectively. It is to be expected that these latter constants will be considerably larger than their respective primary constants, otherwise the efficiency of the enzyme will be very much impaired.

The probability that molecules of the enzyme will form the primary complexes with the substrates is the probability of finding substrate 1 at site 1, and substrate 2 at site 2, simultaneously. The fraction of the enzyme molecules having substrate 1 at site 1 in the absence of substrate 2 is

$$f_1 = \frac{S_1}{K_{1,1}+S_1} = \frac{S_1/K_{1,1}}{1+S_1/K_{1,1}},$$

where S_1 is the concentration of substrate 1. In the presence of substrate 2, the probability of finding substrate 1 at site 1 is

$$F_1 = \frac{S_1/K_{1,1}}{1+\dfrac{S_1}{K_{1,1}}+\dfrac{S_2}{K_{2,1}}},$$

where S_2 is the concentration of substrate 2.

$$\frac{1}{v} = \frac{1}{V}\left[\left(1+\frac{K_{ATP}}{[ATP]}\right)\left(1+\frac{K_{AMP}}{[AMP]}\right)\right]$$

$$= \frac{1}{V}\left[1+\frac{K_{ATP}}{[ATP]}+\frac{K_{AMP}}{[AMP]}+\frac{K_{ATP}\cdot K_{AMP}}{[ATP]\,[AMP]}\right] \qquad (10)$$

$$\text{and} \quad \frac{1}{vi} = \frac{1}{V}\left\{1+\left[\frac{K_{ATP}}{[ATP]}+\frac{K_{AMP}}{[AMP]}+\frac{K_{ATP}\cdot K_{AMP}}{[ATP]\,[AMP]}\right]\left[1+\frac{[ADP]}{K_2}\right]\right\}. \qquad (10a)$$

Similarly,

$$F_2 = \frac{S_2/K_{2,2}}{1+\frac{S_2}{K_{2,2}}+\frac{S_1}{K_{1,2}}}.$$

The probability of the simultaneous presence of substrates 1 and 2 in their primary places is F_1F_2, which is also the ratio v/V. Since this will be the fraction of maximum velocity, it can be seen that V is reached when $F_1F_2 \to 1$. Thus

$$\frac{V}{v} = \frac{1}{F_1F_2} = \left\{1+\frac{K_{2,2}}{S_2}\left[1+\frac{S_1}{K_{1,2}}\right]\right\} \times \left\{1+\frac{K_{1,1}}{S_1}\left[1+\frac{S_2}{K_{2,1}}\right]\right\}. \quad (11)$$

An equivalent equation has been derived by Laidler & Socquet (1950). To observe experimentally the effects of the double affinity of substrate 1 for sites 1 and 2, let $S_2 = K_{2,2}$ ($K_{2,2}$ being the Michaelis constant for its own site). At this concentration, the ratio $S_2/K_{2,1}$ can be neglected, since $K_{2,2} \ll K_{2,1}$. Equation (11) then becomes

$$\frac{V}{v} = \left[2+\frac{S_1}{K_{1,2}}\right]\left[1+\frac{K_{1,1}}{S_1}\right] = \left[2+\frac{S_1}{K_{1,1}}\left(\frac{K_{1,1}}{K_{1,2}}\right)\right]\left[1+\frac{K_{1,1}}{S_1}\right].$$

Let $K_{1,1}/S_1 = x$ and $K_{1,1}/K_{1,2} = R$, then

$$\frac{V}{v} = \left[2+\left(\frac{K_{1,1}/K_{1,2}}{x}\right)\right][1+x] = 2(1+x)+R\left(1+\frac{1}{x}\right). \quad (12)$$

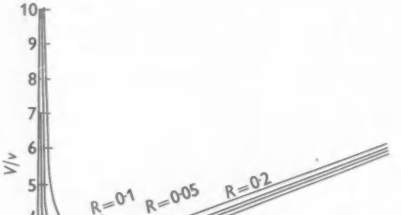

Fig. 8. Plot of V/v against $K_{1,1}/S_1$ for various values of R (see text).

Fig. 8 shows the family of curves obtained by plotting V/v against $K_{1,1}/S_1$ for different values of R, as calculated from equation (12).

It is clear that as the substrate concentration is increased the velocity increases to a maximum. Beyond this point the velocity decreases as the substrate begins to saturate the second site on the enzyme, with resulting inhibition.

Such an effect may be tested for experimentally by plotting $1/v$ against $1/S$ for each substrate, while the other substrate is present at a concentration near its K_m value (that is, its primary association constant).

Kinetics of the reverse reaction; results. (*a*) Inhibition of reverse reaction by ADP.

At pH 7·5 and 30°, 0·5 mM-ADP ([ADP] in equations 10 and 10*a*) caused 24 % inhibition of the reverse reaction in the presence of 0·6 mM-AMP ([AMP]), 0·6 mM chromatographically pure ATP ([ATP]), 0·002 M-$MgCl_2$ and 75 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer. From equations (10) and (10*a*) it was calculated that for a value of K_2 = 0·2 mM-ADP (derived from equation 9) an inhibition of 61 % was to be expected, given that K_{ATP} = 0·3 mM and K_{AMP} = 0·5 mM (these values are given in the results below). If the value K_2 = 1·6 mM-ADP (Callaghan, 1957*b*) was used instead, the theoretical inhibition would be 17 %.

The observed inhibition of 24 % more closely approximates to the last-mentioned value, and the discrepancy may be due to our inability to measure the initial reaction velocities with greater precision. This evidence suggests that it is unnecessary to assume the existence of a second binding constant of low value (K_2) for ADP.

(*b*) Measurement of K_m values for ATP and AMP.

Figs. 9 and 10 show the results of experiments where the velocity of the reverse reaction was measured at various concentrations of one substrate, while the other substrate was initially present at a concentration near its K_m value. The reciprocal plots showed K_m values for ATP and AMP of 0·3 mM and 0·5 mM respectively. In both figures the highest substrate concentration was such that $K_{1,1}/S_1$ was 0·05 (compare with Fig. 8). It will be seen that there was no decrease in reaction velocity at the highest substrate concentrations.

Thus if two sites are present on the enzyme, each site is highly specific for its own substrate. In numerical terms, $K_{1,2}$ must be at least one hundred times as large as $K_{1,1}$ (see Fig. 8, for curve where R = 0·01).

Since it has already been indicated that there is probably only one K_m value for ADP, the simplest explanation of these facts is that myokinase has one site which adsorbs all three substrates in turn.

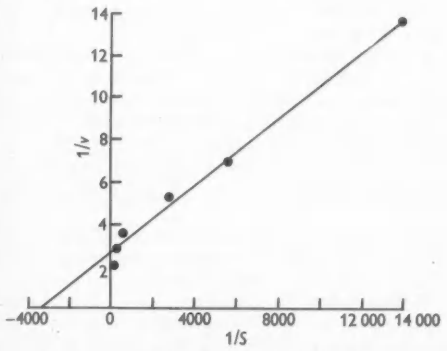

Fig. 9. Lineweaver–Burk plot for the velocity of myokinase reverse reaction at 30°, when the concentration of ATP is varied while AMP is present at its K_m concentration. At zero time, in a total volume of 1·6 ml., the constituents present in the reaction mixtures were: 0·5 mM-AMP; ATP varied from 7·2 mM to 0·072 mM, as indicated on the Figure; 2 mM-$MgCl_2$; 30 mM-tris buffer, pH 7·5; 1 μg. of myokinase. After incubation for 2 min. the reaction was stopped by the addition of 0·1 vol. of 30% (w/v) trichloroacetic acid. Ordinate shows reciprocal of velocity ($1/v$) in μmoles of ATP disappearing and the abscissa shows reciprocal of molar ATP concentration ($1/S$).

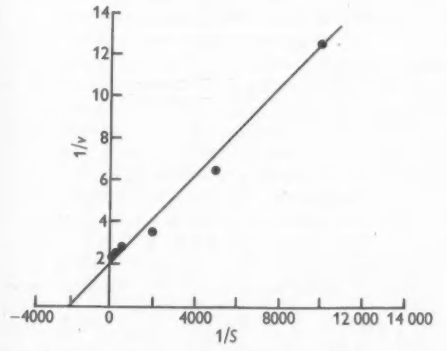

Fig. 10. Lineweaver–Burk plot for the velocity of myokinase reverse reactions at 30° when the concentration of AMP is varied while ATP is present at a concentration near its K_m value. At zero time, in a total volume of 1·6 ml., the constituents in the reaction mixtures were: 0·72 mM-ATP; AMP varied from 10 mM to 0·1 mM as indicated in the Figure; 2 mM-$MgCl_2$; 30 mM-tris buffer, pH 7·5; 1 μg. of myokinase. After incubation for 2 min., the reaction was stopped by the addition of 0·1 vol. of 30% (w/v) trichloroacetic acid. Ordinate shows reciprocal of velocity ($1/v$) in μmoles of ATP disappearing, and the abscissa shows reciprocal of molar AMP concentration ($1/S$).

DISCUSSION

From the results presented in this paper it appears that myokinase does not react with its substrates to form an intermediate phosphorylated enzyme, such as has been described by Sidbury & Najjar (1957) for phosphoglucomutase. Whereas these authors crystallized the phosphorylated phosphoglucomutase, the crystalline preparation of myokinase (Noda & Kuby, 1957*b*) contained only 0·044% of phosphorus in ashed specimens. This is less than 1 g. of phosphorus/21 000 g. of enzyme, and, as was pointed out earlier, traces of nucleotide are strongly bound to the enzyme. This may account for the traces of phosphorus present.

Myokinase belongs to that group of phosphokinases previously studied by Cohn (1953, 1954) and Harrison, Boyer & Falcone (1955), which show similar characteristics of phosphate transfer with respect to the position of cleavage of the phosphate bond. Within this group of phosphokinases, unequivocal evidence for the formation of a phosphorylated enzyme intermediate has not been obtained. Detailed studies of the mechanism of action of pyruvate kinase (Boyer & Harrison, 1954), acetokinase (Rose, Grunberg-Manago, Korey & Ochoa, 1954) and glutamine synthetase (Kowalsky, Wyttenbach, Langer & Koshland, 1956; Boyer, Koeppe & Luchsinger, 1956) have indicated that it is not essential to postulate the formation of this type of intermediate and, for pyruvate kinase, the evidence is against it.

The detailed mechanism of myokinase action is envisaged as follows. The enzyme has a single site which binds specifically one molecule of substrate. On contact with another substrate molecule reaction takes place (equations 4*a* and 4*b*). This contact of the E–S complex with the second substrate molecule may be envisaged as involving a simple collision or, more likely, a binding with Michaelis constant much lower than the one of the first molecule and also involving a much smaller interaction energy and dependent on the previous formation of the E–S complex.

The fact that AMP has approximately equal K_i and K_m deprives this nucleotide of any specific significance as an inhibitor. However, the results obtained in the dialysis and electrophoresis experiments indicate that strong binding of AMP by the enzyme may occur. The relation of this to the specific catalytic binding is not clear.

The following facts summarize the case for a one-site theory of myokinase action: (1) There appears to be only one binding constant for ADP, and calculation indicates that K_m ADP = K_i ADP. (2) Whereas both ATP and AMP inhibit the reaction 2 ADP $\rightarrow$ ATP + AMP in a competitive manner they do not inhibit each other's binding in

the reverse reaction. There are no secondary binding constants for ATP and AMP; the primary association constants are the same, within experimental error, as their inhibitor constants.

K_m ATP = 0·3 mM, K_i ATP = 0·32 mM,

K_m AMP = 0·5 mM, K_i AMP = 0·5 mM.

The potent non-competitive inhibition of myokinase by *p*-chloromercuribenzoate, and the action of this inhibitor in causing the loss of substrate from solutions of enzyme during dialysis, indicate that the SH groups of the enzyme are essential for catalytic activity. It is possible that the attachment of *p*-chloromercuribenzoate to SH groups in the vicinity of the active centre of the enzyme may sterically hinder the adsorption of substrate.

Since none of the other nucleotides tested, with the exception of adenosine monosulphate, inhibited the enzyme, it seems that the intact adenosine phosphate molecule is necessary for attachment to the enzyme. Inosine monophosphate, for example, differs only from AMP by the lack of an amino group on the purine ring, whereas adenosine is AMP minus the phosphate.

The inhibition by adenosine monosulphate suggests that the sulphate radical attaches to a group which is also available to the phosphate group. The amino group on the purine ring was previously stated to be necessary for the binding of ATP and ADP to myokinase by Bendall (1954), and Krebs & Hems (1953) reported that myokinase did not catalyse transphosphorylations involving inosine phosphates.

SUMMARY

1. A phosphorylated enzyme intermediate could not be detected when purified myokinase was incubated with adenosine triphosphate (ATP) and AT$\beta\gamma^{32}$P, comparable concentrations of enzyme and substrate being used, and the enzyme was separated from the substrate by dialysis or zone electrophoresis.

2. The K_m and K_i values for the substrate ATP are 0·3 mM and 0·32 mM respectively. For adenosine diphosphate (ADP), $K_m = K_i = 1{\cdot}58$ mM. For adenosine monophosphate (AMP), the K_m and K_i values are 0·5 mM.

3. Kinetic studies showed that ATP and AMP inhibited the forward reaction competitively.

4. A one-site theory for the mechanism of action of myokinase is proposed and discussed, evidence being brought to show that there is no demonstrable second site on the enzyme for any of the substrates. A theoretical treatment is given for the detection of secondary association constants.

5. *p*-Chloromercuribenzoate inhibits myokinase non-competitively ($K_i = 0{\cdot}015\ \mu$M) and adenosine 5′-monosulphate inhibits myokinase competitively ($K_i = 18{\cdot}6\ \mu$M).

6. Other nucleotides tested did not show detectable inhibition of myokinase, and aniline and benzoate were without effect. Apparently the amino and phosphate groups as well as the adenine ring are specifically required for enzyme activity, although the sulphate group may be substituted for phosphate for purposes of contact with the active centre.

7. A trace of nucleoside monophosphate kinase activity was detected in our myokinase preparations.

8. Myokinase is activated by Mg^{2+} ions. Optimum activation occurs when the Mg^{2+} ion concentration is of the same order as the substrate concentration (0·01 M).

9. The enzyme is inhibited 56–62% by 0·02 M-sodium fluoride.

The authors wish to thank Professor Kunio Yagi for a gift of adenosine 5′-monosulphate. A personal grant from the Medical Research Council to one of the authors (O.H.C.) is gratefully acknowledged. Some of the results given in this paper formed part of a thesis presented by O. H. Callaghan in part fulfilment of the requirements for the degree of Ph.D. at the University of Sheffield.

ADDENDUM

After this paper was written the recent article of Noda [*J. biol. Chem.* (1958) **232**, 237] came to our notice. An important point of difference from the kinetic data reported here was the value of the inhibitor constant for ADP reported by Noda as 0·33 mM, whereas ours appears similar to the $K_m = 1{\cdot}6$ mM. Moreover, Noda obtained a linear plot of $1/v$ against $1/[\text{ADP}]^2$. Such a linear plot would not fit either a one-site ($K_2 = 0$) or two equivalent sites ($K_1 = K_2$) theory as indicated by equation (8). However, over a restricted range of substrate concentration such a plot would be approximately linear where $K_1/\text{ADP} \gg 1$ if two sites of equal K_m were present. On the one-site theory, on the other hand, the plot of $1/v$ against $1/[\text{ADP}]^2$ would yield a curve concave towards the latter axis. In fact it appears to us that the curve of Fig. 6 (for AMP = 0) in Noda's paper shows such behaviour.

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α-1:4-Glucosans

10. GLYCOGEN STRUCTURE AND *RIGOR MORTIS* IN MAMMALIAN MUSCLES*

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Numerous chemical studies have demonstrated that glycogens from various sources may differ in such characteristics as molecular weight, average chain length and in the relative number of D-glucose residues in external and internal chains (Manners, 1957). That constitutional differences in the glycogen molecule have metabolic significance has been indicated by Stetten, Katzen & Stetten (1956), who showed that in the rat and rabbit the larger molecules of muscle glycogen are more reactive, and by Stetten & Stetten (1955), who found that D-glucose residues located peripherally in the chains have a higher rate of turnover than those more centrally situated. Another aspect of the apparent physiological inhomogeneity of mammalian-muscle glycogen has arisen during investigations on post-mortem glycolysis, and on the concomitant onset of *rigor mortis*. In general, provided that pre-slaughter stress has not seriously depleted glycogen reserves, post-mortem glycolysis will continue until pH 5·4–5·5 is reached in the muscles, at which the enzyme systems producing lactic acid are inactivated and some residual glycogen may be found. At a final pH of 5·9, 0·6–1 % of glycogen frequently remains in the psoas and diaphragm muscles of the horse (Lawrie, 1955), and 0·6 % at a final pH of 6·1 in the sternocephalicus muscle of the ox (Howard & Lawrie, 1957). This could signify that the glycogens remaining in such muscles after the completion of *rigor mortis* were either inaccessible or insusceptible to attack during post-mortem glycolysis, or that they might differ in

* Part 9, Eddy, Fleming & Manners (1958).

constitution from the glycogens which predominate in the pre-rigor state. The analytical methods employed in previous papers of this series have therefore been applied to glycogens prepared from some mammalian muscles during the pre- and post-rigor phases.

MATERIALS AND METHODS

Muscles. Samples (0·5–1 kg.) from 7–8-year-old draught horses and 3–4-year-old steers were excised 15–20 min. after death. The following locations were studied: heart (left ventricular wall); diaphragm (junction of pars sternalis with pars costalis); psoas (portion of psoas major at level of sacral vertebrae); longissimus dorsi (l. dorsi; portion posterior to first, second and third lumbar vertebrae); sternocephalicus (entire muscle).

Definition of pre-rigor and post-rigor samples. General features of the time course of *rigor mortis*, and of its associated biochemical changes, in the horse (Lawrie, 1953) and the ox (Howard & Lawrie, 1956, 1957), indicate that muscles from these species, with the exception of horse heart, if sampled when at a pH greater than 6·8 (horse muscles, ox sternocephalicus) or than 6·4 (ox psoas), will then be in the pre-rigor condition provided that they eventually attain a pH of 6·1 (horse muscles, ox sternocephalicus) or 5·7 (ox psoas). Samples of glycogen characteristic of the pre-rigor condition were accordingly prepared from these muscles at a standard time of 1 hr. *post mortem*, when the pH was above the prescribed limit.

Horse heart, however, goes into *rigor mortis* within an hour of death and thus, for practical purposes, yields only post-rigor glycogen, unless special techniques are used.

Samples of glycogen characteristic of the post-rigor phase were prepared from muscle samples held in moist nitrogen at 37° for 5 hr. and thereafter stored for 17 hr. at 0°. The high temperature ensures that conversion of glycogen into lactic acid will occur with the minimum accumulation of hexose phosphate intermediates (Bendall & Davey, 1957); the elapse of 24 hr. from the time of death ensures that the ultimate pH has been attained when the muscle is processed.

pH was determined by glass electrode on samples of muscle, 0·5–1·0 g. being homogenized in 10 ml. of 5 mM-sodium iodoacetate (Bate-Smith & Bendall, 1947).

Preparations of glycogen for analysis. Small pieces cut from the intact muscle were quickly added to hot 30% (w/v) NaOH held in a boiling-water bath (1 g. of muscle to 9 ml. of alkali) and heated until homogeneous. On cooling, ethanol was added to give a final concentration of approximately 50% (v/v). The impure glycogen was centrifuged down after settling overnight and washed with a mixture of 1 vol. of ethanol with 2 vol. of 20% (w/v) NaOH. From this point the procedure followed that described by Somogyi (1957) for the preparation of glycogen free from nitrogen and phosphorus. Each glycogen sample used for structural analysis represented the bulked yields from the muscles of several animals.

Electrophoresis of glycogen. This was kindly examined by Dr D. H. Northcote, using the method of Fuller & Northcote (1956).

Analytical methods

Glucose content. Glycogen was hydrolysed in 2 N-sulphuric acid (0·1%) at 100° for 2 hr. and the glucose content of the hydrolysate determined by the Somogyi (1952) reagent. Hydrolysis of glycogen under the conditions devised by Pirt & Whelan (1951) for the analysis of starch (with 1·5 N-sulphuric acid) gave values 1–2% lower than the above. The slightly stronger acid is necessitated by the greater proportion of α-1:6-glucosidic linkages. A control experiment showed that no destruction of glucose occurred (reducing-power measurements).

Estimation of glycogen. The procedure of Good, Kramer & Somogyi (1933) was employed.

Enzymic degradation and iodine-staining. These analyses were carried out as described by Liddle & Manners (1957), except that, in the α-amylolysis digests, 3·7 units of enzyme/mg. of polysaccharide was used. P_M denotes apparent percentage conversion into maltose. $E_{max.}$ represents the extinction (absorption value) of the iodine-stained polysaccharide at the $\lambda_{max.}$.

Glycogen value. The interaction of glycogen and concanavalin-A prepared from jack-bean meal was examined under the conditions of Cifonelli, Montgomery & Smith (1956), except that extinctions were measured on a Unicam SP. 500 spectrophotometer. The extinction given under these conditions by 1 mg. of glycogen was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1·00) kindly provided by Professor F. Smith. Amylopectins did not react under these conditions (see Calderbank, Kent, Lorber, Manners & Wright, 1960).

Periodate oxidation. The horse l. dorsi (post-rigor) muscle glycogen (about 250 mg.) was oxidized with a mixture of 20 ml. of 8% (w/v) sodium metaperiodate solution and 80 ml. of 5% (w/v) potassium chloride solution at room temperature, as described by Bell & Manners (1952). A second sample (about 100 mg.) was oxidized with sodium metaperiodate at 2° under the conditions of Manners & Archibald (1957).

The remaining glycogens (about 75 mg.) were oxidized with a suspension of potassium metaperiodate (5 ml. of sodium metaperiodate and 20 ml. of potassium chloride solutions) at room temperature. The oxidation was completed after 9–10 days, and 10 ml. samples were removed for analysis of formic acid after 10 and 12 days. A reagent control was also prepared and analysed.

RESULTS

Glycolytic criteria of horse and ox muscles

Characteristic pre- and post-rigor values for the pH and glycogen concentration in the muscles studied are given in Table 1. There is a considerable concentration of residual (or post-rigor) glycogen in all the muscles except horse heart. In horse l. dorsi and ox psoas, where the final pH is about 5·5, inactivation of glycolytic enzymes presumably accounts for the unattacked glycogen. This reason cannot automatically be adduced for horse psoas and diaphragm and ox sternocephalicus muscles, since the final pH is 5·8–6·0.

Table 1. *Characteristic* pH *values and glycogen contents of muscles investigated*

Values in parenthesis indicate the number of specimens examined. Initial pH and initial glycogen values were obtained 1 hr. *post mortem*.

Muscle	Initial pH	Final pH	Initial glycogen (pre-rigor) (mg./100 g.)	Residual glycogen (post-rigor) (mg./100 g.)
Horse heart	6·14±0·07 (4)	5·86±0·06 (4)	723± 64 (4)	79± 45 (4)
Horse psoas*	6·77±0·04 (6)	5·85±0·06 (6)	1229±190 (6)	606±143 (6)
Horse diaphragm	6·92±0·03 (6)	5·86±0·04 (6)	1883±127 (6)	1184± 43 (6)
Horse l. dorsi	6·87±0·05 (6)	5·45±0·08 (6)	2216±125 (6)	1179±101 (6)
Ox psoas†	6·41±0·07 (12)	5·53±0·07 (12)	772±125 (12)	205± 43 (9)
Ox sternocephalicus	7·11±0·04 (3)	6·01±0·15 (3)	1333±350 (3)	492±110 (3)

* From Lawrie (1955). † From Howard & Lawrie (1956).

Table 2. *Properties of horse- and ox-muscle glycogens*

$E_{max.}$ represents the extinction at $\lambda_{max.}$ after iodine treatment. ECL represents the average length of the exterior chains (no. of glucose residues removed by β-amylase + 2·5).

Sample	Chain length (average)	Glycogen value	$\lambda_{max.}$ (mμ)	$E_{max.}$	P_M	β-Amylolysis limit (%)	ECL
Horse, l. dorsi pre-rigor	17	0·87	480	0·34	83	53	11–12
Horse, l. dorsi post-rigor	17	0·82	490	0·36	78	47	10
Horse, diaphragm pre-rigor	17	0·81	475	0·31	80	53	11–12
Horse, diaphragm post-rigor	17	0·85	480	0·32	80	51	11
Horse, psoas pre-rigor	16–17	—	490	0·32	79	48	10–11
Horse, psoas post-rigor	17	0·76	490	0·35	76	46	10
Horse, heart, post-rigor	16–17	0·83	470	0·29	78	48	10–11
Ox, psoas pre-rigor	16–17	0·86	490	0·34	82	50	11
Ox, psoas post-rigor	15	—	480	0·30	79	50	10
Ox, sternocephalicus pre-rigor	19	0·78	475	0·27	83	51	12
Ox, sternocephalicus post-rigor	15	0·89	485	0·21	79	44	9

Characterization of purified glycogens

Glucose contents. The glycogen samples had glucose contents in the range 96–100 %, and the following analyses are based on these figures.

Iodine staining. The glycogens stained deep red–brown with iodine, and the spectra showed maximum absorption in the range 470–490 mμ. The $\lambda_{max.}$ values in Table 2 represent the mid-point of a wide absorption peak covering 20–30 mμ. Mammalian-liver glycogens under similar conditions show maximum absorption over the range 450–470 mμ ($E_{max.}$ 0·2–0·3), whereas amylopectins have $\lambda_{max.}$ values of 530–540 mμ and $E_{max.} > 0·6$.

Glycogen values. The glycogen values of the muscle glycogens varied between 0·76 and 0·89, indicating a slightly lower degree of branching than usual. There was no appreciable difference between pre- and post-rigor samples.

Average chain length. Potassium metaperiodate oxidation of horse l. dorsi (post-rigor) muscle glycogen (252 mg.) gave formic acid corresponding to chain lengths of 16·9, 16·6 and 16·6 glucose residues after 9, 10 and 11 days. In a duplicate analysis, the production of formic acid from 251 mg. of this glycogen corresponded to an average chain length of 16·6 glucose residues.

Sodium metaperiodate oxidation of the same glycogen (105 mg.) at 2° for 20 days indicated an average chain length of 16·8 glucose residues.

The remaining glycogens were analysed after 10–12 days' oxidation; the sodium hydroxide titres after 10 and 12 days were identical. The production of formic acid indicated average chain length values as follows: Pre-rigor samples: horse l. dorsi, 16·8; horse diaphragm, 16·8; horse psoas, 16·5; ox sternocephalicus, 18·6; ox psoas, 16·5. Post-rigor samples: horse diaphragm, 16·6; horse psoas, 17·1; horse heart, 16·5; ox sternocephalicus, 14·7 and 15·0 in duplicate analyses; ox psoas, 16·5. These values are considered to be accurate to within ±0·5 glucose residue. These results, to the nearest whole number, are summarized in Table 2.

β-Amylolysis limits. Incubation of the glycogens at pH 4·6 and 35° with barley β-amylase resulted in 44–53 % conversion into maltose.

α-Amylolysis limits. On incubation with purified salivary α-amylase at pH 7·0 and 35° for 24 hr., P_M values in the range 76–83 were obtained. The majority of these figures are significantly higher than those observed (approx. 75 %) with mammalian-liver glycogens (average chain length 13–14), and indicate a slightly lower degree of branching.

Electrophoresis. On zone electrophoresis (glass-fibre paper) in 0·1 M-sodium borate buffer, pH 9·3, at 2000 V and 140 mA for 90 min., the glycogens moved 6–8 cm. as one component. The apparent homogeneity of the muscle glycogens may be contrasted with the studies of Lewis & Smith (1957), who found that a variety of glycogens gave two distinct components on electrophoresis in 2N-sodium hydroxide solution. There were small differences between the mobilities of the pre- and post-rigor samples examined, but these were not in a consistent direction.

The properties of the mammalian-muscle glycogens are summarized in Table 2.

DISCUSSION

Extraction and isolation of glycogen from animal tissues

The conventional solvents for the extraction of glycogen from animal tissues are hot water, cold dilute aqueous (5–10%) trichloroacetic acid and hot concentrated aqueous (30%) potassium or sodium hydroxide (Pflüger method). The last-named method has been criticized by many workers (e.g. Meyer & Jeanloz, 1943; Stetten, Katzen & Stetten, 1958; Bryce, Greenwood & Jones, 1958) as causing considerable degradation of the glycogen molecules. There is ample evidence to show that glycogen is degraded by hot dilute alkali (e.g. Greenwood & Manners, 1957; Stetten *et al.* 1958), but the available data indicate that hot concentrated alkali does not cause a progressive reduction in molecular weight. Thus Staudinger (1948) found that treatment of guinea-pig liver and muscle glycogen with 15 or 30% potassium hydroxide solution at 100° for 1 hr. did not alter the molecular weight (light-scattering measurements). Bryce *et al.* (1958) showed that the sedimentation constant of rabbit-liver glycogen was not progressively reduced after treatment for 3 hr. with hot 30% potassium hydroxide. Furthermore, Cori & Cori (1958) reported that the distribution of sedimentation constants of glycogen was unaffected after treatment with hot concentrated alkali. This marked difference in the degradative action of dilute and concentrated alkali may be related to the low solubility of oxygen in the latter.

The Pflüger method has therefore been used throughout the present study since it gives a high yield of protein-free glycogen (extraction of muscle tissue with hot water or trichloroacetic acid is extremely inefficient), and does not appear to cause appreciable degradation of the constituent glucopyranosidic linkages.

Previous studies on muscle glycogen

Although the molecular structures of mammalian liver and invertebrate glycogens have been extensively studied (Abdel-Akher & Smith, 1951; Manners, 1957), considerably less attention has been paid to mammalian-muscle glycogens. This may be attributed to the relative difficulty in obtaining adequate samples of tissue, and their low glycogen contents (usually $< 1\%$). The presence of glycogen in muscle tissue was first noted by Sanson (1857), but some 80 years elapsed before the first chemical investigations were reported (Young, 1937; Bell, 1937). These indicated that rabbit-muscle and liver glycogens had similar properties, and that a sample of horse-muscle glycogen (pooled tissues) had an average chain length of 11–12 by methylation. A summary of the results of other end-group assays of muscle glycogens is given in Table 3. These differ markedly from those reported in Table 2. Furthermore, the β-amylolysis limits ($42 \pm 4\%$) are significantly lower than those of our present analyses.

Molecular structure of pre- and post-rigor muscle glycogens

Although glycogens have been isolated with average chain length values ranging from 6 to 18, the majority of samples have values of 12 ± 2 (Manners, 1957). The most noticeable feature of the mammalian-muscle samples as a whole (and, in particular, of the sternocephalicus pre-rigor glycogen) is

Table 3. *Properties of some mammalian-muscle glycogens*

Methods of assay are indicated by: (m) methylation; (p) periodate oxidation; (e) enzymic.

Sample	Chain length (average)	β-Amylolysis limit (%)	References
Horse	11–12 (m, p)	42	Bell (1937); Bell & Manners (1952)
Human	11 (p)	—	Halsall, Hirst & Jones (1947)
	12 (p)	41	Bell & Manners (1952)
	11 (p)	40	Liddle & Manners (1957)
Rabbit	13 (p)	—	Halsall *et al.* (1947)
	11–13 (m, p)	45	Bell (1948*a*); Bell & Manners (1952)
	11 (p)	39	Liddle & Manners (1957)
	15 (e)	—	Illingworth, Larner & Cori (1952)
	13 (p)	46	Manners & Wright (unpublished work)

therefore the significantly lower degree of branching, as determined by potassium periodate oxidation. This method is well established in carbohydrate chemistry for the analysis of non-reducing polysaccharides, and the possibility of any inherent error is reduced by the close agreement reported by other workers between methylation, enzymic and periodate-oxidation analyses of the same samples.

The potassium periodate-oxidation results in Table 2 are supported by the glycogen and P_M values.

The interaction of concanavalin-A and glycogen was first examined in detail by Cifonelli *et al.* (1956), who noted that the glycogen values of various glycogens were increased by β-amylolysis. They concluded that the reaction mainly involved the interior chains. However, it seems probable that other factors are also significant since measurement of the glycogen values of various human glycogens (from cases of glycogen-storage disease) have indicated an approximate relationship with the degree of branching (Calderbank *et al.* 1960). The glycogen values of the horse-muscle glycogens are significantly lower than those of 12–14 unit glycogens and are therefore consistent with the lower degree of branching and the relatively longer exterior chains.

The extent of degradation by α-amylase may, to a first approximation, be related to the degree of branching in the polysaccharide, since the interchain linkages and certain adjacent α-1:4-glucosidic linkages are resistant to enzyme action (Whelan & Bines, 1955). The observed trend of P_M values for the muscle glycogens is fully in accord with the significantly lower degree of branching, as the values are intermediate between those of a normal glycogen (average chain length 14, P_M 75) and an amylopectin (average chain length 22, P_M 89).

The iodine-staining power of mammalian-muscle glycogen is appreciably greater than that of liver or invertebrate glycogens (Manners, 1957). However, this feature of muscle glycogens, which has been noted by other workers (Young, 1937; Bell, 1948*b*), is not apparently directly related to the molecular structure. The iodine-staining data in Table 2 serve to characterize the muscle polysaccharides as 'glycogens' rather than 'amylopectins'. For example, the amylopectin component of malted barley starch has an average chain length of only 18 (Aspinall, Hirst & McArthur, 1955) but has $\lambda_{max.}$ 540 mμ and $E_{max.}$ 0·77 (unpublished data).

The results recorded in Tables 1 and 2 show that the glycogens remaining after the development of *rigor mortis* in horse muscles, and in ox psoas, do not differ significantly in molecular structure from the initial pre-rigor polysaccharides. On the other hand, in ox sternocephalicus muscle there is a significant shortening of the outer chains, from 12 to 9 glucose residues, during post-mortem glycolysis.

Biochemical and physiological implications

The biochemically significant quantity of glycogen which remains unattacked by the glycolysis system at a final pH of 6·0 in ox-sternocephalicus muscle appears to represent a molecular species having a shorter average external chain length than that initially present. In this instance, the presence of residual glycogen at a high final pH may be rationalized at the chemical level. Although in horse psoas and diaphragm residual glycogen at a high final pH is found, this does not apparently differ from the pre-rigor glycogens in these muscles. The pre- and post-rigor glycogens in these muscles need not be physiologically equivalent, however, since fractionation of a given glycogen sample can yield species of different molecular weight but similar branching characteristics (Stetten & Stetten, 1958). This may have physiological importance as glycogen of high molecular weight appears to be preferentially degraded *in vivo* and *in vitro* by muscle phosphorylase (Stetten *et al.* 1958; Larner, Ray & Crandall, 1956) and to exchange its glucose units more vigorously with the environment than that of low molecular weight (Stetten *et al.* 1956). In general, post-rigor glycogens would thus be expected to have a low molecular weight and a more compact molecular-weight distribution. If this is so, there would seem to be no relationship between the degree of branching and molecular size in the horse-muscle glycogens examined.

Dr W. A. J. Bryce has kindly determined the sedimentation constants of the l. dorsi muscle glycogens; the polysaccharides were polymolecular, values of 96 and 80×10^{-13} c.g.s. unit being obtained for the major components of the pre- and post-rigor samples, corresponding to molecular weights of 4·4 and $3·7 \times 10^6$ [assuming a diffusion constant of $1·50 \times 10^{-7}$ (Bell, Gutfreund, Cecil & Ogston, 1948)]. These results are qualitatively in agreement with the above suggestion.

In view of the similar average chain length values of the pre- and post-rigor glycogens from horse muscle and ox psoas, and assuming that the major pathway of glycogen breakdown involves phosphorylase, it is clear that the relative activity of phosphorylase and amylo-1:6-glucosidase is largely unaltered during post-mortem glycolysis. This may be contrasted with other metabolic conditions, e.g. glycogenesis, in which an alteration in activity is manifested by the presence of glycogen with relatively short exterior chains and a higher degree of branching (Manners, 1957). On the other hand, with ox-sternocephalicus muscle, the significant shortening of the outer chains from 12 to 9 glucose residues may indicate either a partial inactivation

of the debranching enzyme, or *in vivo* heterogeneity with respect to branching characteristics.

The concentration of glycogen in muscle has hitherto been recognized as an aspect of functional specialization, since it tends to be high in so-called white muscles, which derive energy for short bursts of activity by anaerobic glycolysis, and low in so-called red muscles, which are equipped for sustained energy production by respiration. That this relatively simple concept might be inadequate was indicated by the work of Bloom, Lewis, Schumpert & Shen (1950) and Kits van Heyningen & Kemp (1955). It now appears that distinct molecular species of glycogen, exhibiting different susceptibilities to glycogen breakdown, may occur in a given muscle. This presumably reflects some further aspect of functional specialization.

SUMMARY

1. The molecular structure of glycogen isolated by the Pflüger method from some mammalian muscles during the pre- and post-phases of *rigor mortis* has been examined by chemical and enzymic methods.

2. Glycogen from pre- and post-rigor horse-muscle tissues has an average chain length of approximately 17 glucose residues. This indicates a significantly lower degree of branching than is present in other samples of mammalian and invertebrate glycogen.

3. The glycogen isolated from ox-sternocephalicus muscle after the onset of *rigor mortis* has significantly shorter exterior chains than that isolated from the pre-rigor muscle. This could indicate either partial inactivation of the enzyme system catalysing the glycogen $\rightleftharpoons$ glucose 1-phosphate interconversion or a true heterogeneity of the glycogen in this muscle *in vivo*.

4. The chemical and physiological significance of these observations is discussed.

5. The possibility of the degradation of glycogen during alkaline extraction is considered.

We wish to thank Professor E. L. Hirst, F.R.S., and Dr F. A. Isherwood for their interest in this work, which for one of us (R. A. L.) formed part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. One of us (A. W.) is indebted to the Department for a maintenance allowance. Mr W. A. Deer gave technical assistance.

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Determination of the Reduced and Oxidized Pyridine Nucleotides in Animal Tissues

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The determination of the concentration of the reduced and oxidized pyridine nucleotide in biological material is a matter of interest, because of the key role of these substances in intermediary metabolism. When the work reported in this paper was begun a sensitive method suitable for the determination of $0{\cdot}3 \times 10^{-9}$ mole in 1 ml., elaborated by Glock & McLean (1955), was available but difficulties were encountered in preparing the diphosphopyridine nucleotide-linked cytochrome *c* reductase required for the determination. After the publication of the fluorimetric procedure of Lowry, Roberts & Kapphahn (1957) it was decided to employ this method but it was found necessary to introduce modifications.

The concentrations of reduced coenzymes in liver reported by Lowry *et al.* were considerably lower than the corresponding data presented by Glock & McLean, and a comparison of the extraction procedures used by these two groups of workers showed the method of Glock & McLean to be superior in that their alkaline extract (pH about 13) contained more reduced coenzyme than the extract of Lowry *et al.* made at pH 8. Accordingly, an extraction procedure similar to that of Glock & McLean was adopted. A further modification of the method of Lowry *et al.* was necessary because of the oxidation of the reduced coenzymes during subsequent manipulation of the alkaline extract. The rate of oxidation is very rapid after adjustment of the alkalinity to pH 8·0. This invalidates the assumption that all the coenzymes in this extract are in the reduced form. The error introduced by this oxidation was more serious when the extracts were stored (even at $-15°$) before estimation. However, by combining the selective destruction of the oxidized coenzymes by alkali and of the reduced coenzymes by acid (see Schlenk, 1951) with dehydrogenase reactions specific for diphosphopyridine nucleotide and triphosphopyridine nucleotide respectively, a satisfactory procedure for the estimation of both oxidized and reduced di- and triphosphopyridine nucleotides in concentrations as low as 1·5 μM was obtained.

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Whilst this work was in progress, another method was published by Jacobson & Astrachan (1957). Their procedure requires the preparation of five enzymes and the sensitivity is less than that of the other methods.

This paper presents the results obtained in an investigation of a number of factors influencing the precision and accuracy of the method; and a series of values for the level of coenzymes in several tissues from different animals and for the levels of oxidized and reduced coenzymes during the incubation of tissue preparations *in vitro*.

EXPERIMENTAL

Diphosphopyridine nucleotide (DPN) was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. It contained 91% of DPN (by the alcohol dehydrogenase method of Racker, 1950) and no triphosphopyridine nucleotide (TPN). TPN was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. It contained 92% of TPN when analysed by fluorimetry against a DPN standard solution prepared from the DPN described above.

Reduced diphosphopyridine nucleotide (DPNH) was obtained from Sigma Chemical Co. and contained 62% of DPNH when analysed by fluorimetry. Reduced triphosphopyridine nucleotide (TPNH) was obtained from Sigma Chemical Co. and contained 27% of TPNH when analysed by fluorimetry.

Glucose 6-phosphate dehydrogenase (G 6-P dehydrogenase) was prepared by the method of Kornberg (1950) and stored in the lyophylized form at $-20°$. Alcohol dehydrogenase was prepared from baker's yeast by the method of Racker (1950). The crystalline enzyme was stored in half-saturated $(NH_4)_2SO_4$ at $-20°$.

Nicotinamide and ethylenediaminetetra-acetic acid (disodium salt; EDTA) were obtained from British Drug Houses Ltd. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris) was obtained from Sigma Chemical Co. Marsalid phosphate and isoniazid were kindly supplied by Roche Products Ltd., Welwyn Garden City, Herts. Acetaldehyde was redistilled before use and 10 mM-solutions were prepared as required.

Glucose 6-phosphate (G 6-P) was obtained as the barium salt heptahydrate from British Drug Houses Ltd. The barium was removed with Dowex-50 H^+ resin and the glucose 6-phosphoric acid neutralized by the addition of $NaHCO_3$ solution. The final strength of the solution was 0·1 M. Hydrogen peroxide (3%, w/v) was prepared by

dilution of 30% (w/v) reagent obtained from British Drug Houses Ltd., and was stored at 2°.

Experimental animals. Rats were of the white Wistar strain. Mice were CBA strain and the New Zealand strain of obese mice (N.Z.O.) was used.

Sheep heart was obtained from the abattoir, the tissue being transferred as speedily as possible to a Dewar flask containing crushed ice.

Preparation of homogenate. Tissues were minced in the Fischer mincer (Établissement Jouan, Paris) and portions of the mince were weighed into cooled beakers and then transferred with cold homogenizing medium into a cold stainless-steel homogenizer of the Potter–Elvehjem type. The pestle was rotated at 2000 rev./min.

Preparation of minces. Tissues were minced in the Fischer mincer, portions were weighed into cooled beakers and suspended in cold medium.

Preparation of chopped tissue. The mechanical tissue chopper (McIlwain & Buddle, 1953) was used. Prisms of dimensions 0·35 mm. × 0·35 mm. × 3 mm. were prepared and suspended in cold medium. The entire preparation was carried out in the cold room at 2°. The above-mentioned preparations were suspended in the medium (without calcium) with added phosphate buffer, pH 7·4, of Krebs & Eggleston (1940).

Measurement of fluorescence. The fluorescence developed from samples containing pyridine nucleotides was measured in the Farrand fluorimeter model A, Farrand Optical Co. Inc., New York 70, N.Y. The primary filter was Farrand no. 5860 and the secondary filter comprised Farrand no. 3389 and 4308 together with Wratten no. 2B. The instrument was regularly standardized against a solution of quinine sulphate at 0·1 mg./l. of 0·1 N-H_2SO_4.

Extraction procedures

Whole tissue. Two small glass homogenizers containing the following extracting fluids were heated in boiling water. Acid medium: 5 ml. of 0·1 M-tris–HCl buffer, pH 8·2; 2 ml. of water; 1 ml. of N-HCl. Alkaline medium: 5 ml. of tris–HCl buffer; 2 ml. of water; 1 ml. of N-NaOH.

The test animal was killed, the liver removed as quickly as possible and two portions of 300–500 mg. were weighed and placed in the hot extracting fluids. The time from killing the animal to placing the samples in the medium was less than 2 min.

During the first heating for 10 sec., the liver was broken into small pieces with a glass rod and then homogenized for about 30 sec., after which it was briefly heated again. After a total heating time of 2 min., rapid cooling was achieved by immersion of the homogenizers in liquid air and by stirring the contents until they froze. The mixtures were thawed, and stirred during adjustment to pH 8·0 (checked with indicator papers) with 1 ml. of either N-HCl or N-NaOH. The solutions were transferred to chilled graduated tubes and the volumes made up to 10 ml. The extracts were then centrifuged at 20 000 *g* for 15 min. in a refrigerated centrifuge. It was useful to employ an upper layer of *n*-hexane during centrifuging to prevent retention of particles by fat at the upper surface of the aqueous layer. The supernatant aqueous layers were removed with Pasteur pipettes, immediately frozen and retained for analysis. Subsequently the extract prepared in the acid medium is designated 'acid extract' and that prepared in the alkaline medium 'alkaline extract'.

Homogenate, mince and chopped tissue. For each analysis of a homogenate, mince or chopped tissue, two small conical flasks (about 15 ml. volume) were used. One contained 1 ml. of tris–HCl (0·1 M, pH 8·2) and 0·25 ml. of N-NaOH, the other contained 1 ml. of tris–HCl (0·1 M, pH 8·2) and 0·25 ml. of N-HCl. The flasks were heated in boiling water.

Samples (1 ml.) of tissue preparation were added to the appropriate flasks, which were periodically shaken in the water bath during heating for 2 min. The flasks were removed and cooled rapidly in liquid air until the contents were frozen. The subsequent treatment was similar to that described for whole-tissue extracts.

Solutions required for estimations

Glucose 6-phosphate dehydrogenase. The activity of aqueous solutions of the freeze-dried enzyme was adjusted so that 10 μl. reduced 10^{-8} mole of DPN at pH 8·2 (tris–HCl buffer) in a total volume of 0·5 ml. in 10 min.

Alcohol dehydrogenase. The activity of aqueous solutions of the stock suspension was adjusted so that 10 μl. reduced 10^{-8} mole of DPN at pH 8·2 (tris–HCl buffer) in a total volume of 0·5 ml. in 10 min.

Approximately N-hydrochloric acid and -sodium hydroxide. Solutions of identical normality were prepared. These will be referred to as 'standard' HCl and 'standard' NaOH.

Sodium hydroxide–hydrogen peroxide solution. This was prepared immediately before use by adding 0·1 ml. of the stock 3% H_2O_2 to each 3 ml. of 9 N-NaOH. The appearance of a fine precipitate in this solution did not affect the reproducibility of the results.

Standard DPN solution. A solution (20 μM) of DPN was prepared in tris–HCl buffer, pH 8·2, at a molarity close to that of the test solutions (about 0·03 M).

Analytical procedure

The acid and alkaline extracts from tissue preparations were analysed according to the scheme shown in Fig. 1.

Acid extract. To a sample (0·5 ml.) of the acid extract (solution A, Fig. 1) in a 3 ml. tube was added 10 μl. of G 6-P dehydrogenase solution and 10 μl. of G 6-P. The solutions were mixed and allowed to stand at room temperature (about 20°) for 20 min. before further manipulation.

Alkaline extract. The alkaline extract (solution A′, Fig. 1) contains the coenzymes which in the original tissue preparation were in the reduced form. It was found that a proportion of the reduced coenzymes in this extract became reoxidized during the lengthy manipulations involved in both the extraction and estimation; when storage of this extract was necessary reoxidation continued even at −15°. It was therefore necessary to separate oxidized from reduced coenzymes by treating one sample (0·5 ml.) of the alkaline extract with 30 μl. of standard HCl at room temperature for 5 min. and another sample of 0·5 ml. with 30 μl. of standard NaOH at 60° for 15 min. Then each solution was adjusted to pH 8·2 by the addition of 30 μl. of standard alkali or acid to give solution B (containing oxidized coenzymes) and solution C (containing reduced coenzymes). The solutions were stirred continually during adjustment to pH 8·2.

Solution B. This volume (0·5 ml.) was given the same treatment as the 0·5 ml. sample of 'acid extract' above.

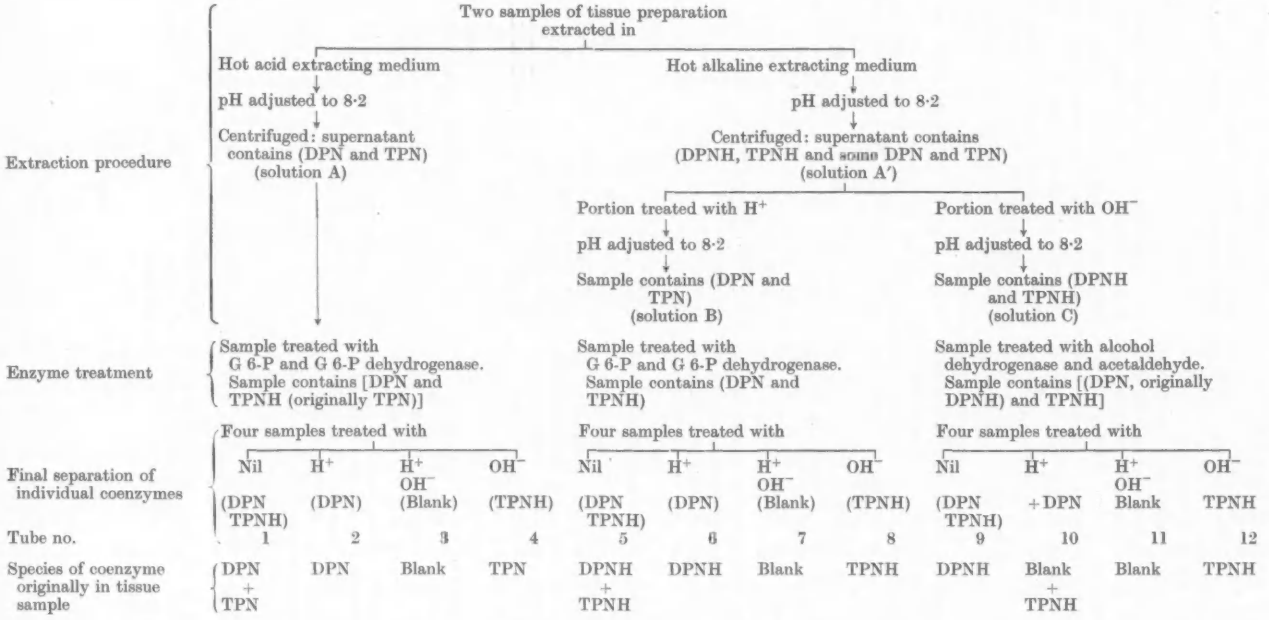
Two samples of tissue preparation extracted in
Hot acid extracting medium
Hot alkaline extracting medium
pH adjusted to 8·2
pH adjusted to 8·2
Centrifuged: supernatant contains (DPN and TPN) (solution A)
Centrifuged: supernatant contains (DPNH, TPNH and some DPN and TPN) (solution A')
Portion treated with H+
Portion treated with OH−
pH adjusted to 8·2
pH adjusted to 8·2
Sample contains (DPN and TPN) (solution B)
Sample contains (DPNH and TPNH) (solution C)
Extraction procedure
Sample treated with G 6-P and G 6-P dehydrogenase. Sample contains [DPN and TPNH (originally TPN)]
Sample treated with G 6-P and G 6-P dehydrogenase. Sample contains (DPN and TPNH)
Sample treated with alcohol dehydrogenase and acetaldehyde. Sample contains [(DPN, originally DPNH) and TPNH]
Enzyme treatment
Four samples treated with
Four samples treated with
Four samples treated with
Nil H+ H+ OH− OH−
Final separation of individual coenzymes
(DPN TPNH) (DPN) (Blank) (TPNH)
(DPN TPNH) (DPN) (Blank) (TPNH)
(DPN TPNH) +DPN Blank TPNH
Tube no.
1 2 3 4 5 6 7 8 9 10 11 12
Species of coenzyme originally in tissue sample
DPN + TPN
DPN
Blank
TPN
DPNH + TPNH
DPNH
Blank
TPNH
DPNH
Blank + TPNH
Blank
TPNH

Fig. 1. Scheme of analytical procedure. Fluorescence was developed in each of the tubes 1 to 12 by the addition of the appropriate amounts of conc. $NaOH–H_2O_2$ mixture. Values obtained in the pairs of tubes 5 and 9, 6 and 10, 8 and 12, are combined. H^+ indicates 'standard' acid, OH^- indicates 'standard' alkali.

Solution C. To this 10 μl. of 0·035 M-EDTA, 10 μl. of alcohol dehydrogenase solution and 5 μl. of 0·01 M-acetaldehyde were added and the mixture was allowed to stand at room temperature for 20 min. before further manipulation.

From this point in the analysis each of the solutions A, B and C received the following identical treatment.

Four samples, each of 25 μl., were placed in fluorimeter tubes. The first of these tubes received no standard acid or alkali. To the second tube 2 μl. of standard HCl was added; to the third tube 2 μl. of standard HCl was added and, at the end of 5 min., 7 μl. of standard alkali; to the fourth tube 5 μl. of standard alkali was added. Both tubes receiving alkali were heated at 60° for 15 min. and then cooled.

To every fluorimeter tube 0·1 ml. of the NaOH–H_2O_2 mixture was added and, after mixing, the tubes were heated in a water bath at 38° for 60 min. It was necessary to use distilled water in the bath to prevent the possible contamination of the outside of the tubes by fluorescent material.

The fluorescence from a known amount of coenzyme was determined by including with each batch of estimations three tubes containing 25 μl. of standard 20 μM-DPN solution. The DPN in one of these samples was destroyed by adding 5 μl. of standard NaOH and heating at 60° for 15 min. The fluorescence developed in this sample was subtracted from the mean value obtained from the other two standard DPN samples.

When values for total oxidized and total reduced coenzyme only were required, the procedure was shortened by treating samples of solutions A and A′ (see Fig. 1) with the concentrated NaOH–H_2O_2 mixture, and fluorescence developed in the usual way. Each determination involved the production of a blank with the standard acid and standard alkali treatment.

Owing to the relatively low concentrations of TPN in the tissues studied, it was sometimes desirable to increase the volume of sample finally analysed from 25 to 75 μl. Certain modifications of the method were then necessary. The quantity of standard acid and alkali added to destroy the coenzymes was increased threefold, and since the final concentration of alkali in the mixtures which were heated at 38° for 60 min. must exceed 6 N, the concentrated NaOH–H_2O_2 mixture was prepared by adding 0·16 ml. of 3% (w/v) H_2O_2 to each 3 ml. of 15 N-NaOH. Analyses of the same solution by the standard method and with this modification gave comparable results.

Blanks. The blanks obtained with a particular tissue were reasonably constant. For example, with the 'acid' extract from rat-liver mince, mean value from twenty-four experiments for the galvanometer readings was 13, with S.D. of 3; and for the alkaline extract was 20, S.D. of 6. Blank values at the upper limit of these ranges were not correlated with a low observed amount of coenzyme, either in simultaneous analyses of the livers of several different animals or in analyses of different extracts of the same liver. Some of the highest total coenzyme concentrations observed were associated with relatively high blank values.

Infrequently, after incubation of tissues, the fluorescence in the blank samples treated with standard acid followed by standard alkali (see Fig. 1) increased greatly (up to four times the original values) and exceeded that found in the samples treated with either standard acid or standard alkali alone. When this occurred, an alternative method for producing the blank was employed, in which the oxidized coenzymes were first reduced enzymically and then all the coenzymes in the sample were destroyed with standard acid.

RESULTS

Recovery of added coenzymes. The recoveries (see Table 1) were determined by adding known amounts of coenzymes to tissue preparations during extraction. The coenzyme was added after heating a suspension of rat-liver mince for 30 sec., and heating was then continued for a further 2 min. before freezing. The endogenous coenzyme was determined in a separate extract.

Factors influencing the sensitivity of the method. The presence of tris–HCl buffer in solutions of the coenzymes reduced the fluorescence developed. Accordingly, all standard coenzyme solutions were made in this buffer.

Lowry *et al.* (1957) have shown that certain compounds decrease the fluorescence developed from coenzyme samples, and note that H_2O_2 reduces this effect. With the NaOH–H_2O_2 mixtures described in this paper, pyruvate, α-oxoglutarate and

Table 1. *Recovery of coenzymes added to rat-liver mince during extraction*

Values given are means ± S.E.M. The amount of coenzyme added was one to three times the endogenous amount.

Coenzyme added	No. of expts.	Recovery (%)
DPN	4	93 ± 6
TPN	5	93 ± 3
DPNH	4	104 ± 5
TPNH	8	88 ± 7

Table 2. *Coenzymes extracted in 'neutral', acid and alkaline media*

The tissue preparation was a 1 in 15 suspension of rat-liver mince (Fischer mincer). The acid and alkaline extractions were performed as described in the Experimental section. The 'neutral' extraction was made in tris–HCl buffer (pH 8·2, 0·1 M), and the supernatant after centrifuging was analysed for total oxidized and reduced coenzymes in the manner described for the neutralized supernatant from the alkaline extract (see Fig. 1 down to solutions B and C).

	Coenzyme content (μm-moles/g. fresh wt.)				
	DPN	TPN	DPNH	TPNH	Total
Acid extract	485	103	—	—	1174
Alkaline extract	—	—	302	284	
'Neutral' extract	445	115	215	114	889

fructose at the concentrations greater than about 5 mM reduced the fluorescence, whereas succinate and citrate did not, i.e. compounds containing keto groups are responsible for the interference. At concentrations less than 5 mM in the final sample, pyruvate and α-oxoglutarate had no effect.

Extraction procedure. A comparison of the extraction made with the acid and alkaline media used by Glock & McLean (1955) and the single extraction in tris–HCl buffer, pH 8·2, used by Lowry *et al.* (1957) showed that the former procedure was to be preferred (see Table 2).

No significant variation was found in the amount of coenzyme extracted during times of heating ranging from 1·5 to 4·5 min. Outside these limits values were lower. As a routine, heating times of 2·0–2·5 min. were used.

The efficiency of the extraction was not affected by homogenizing the tissue preparation during the heating.

Oxidation of reduced coenzymes in neutralized alkaline extracts. It was found that on storage of the neutralized alkaline extracts, considerable oxidation of the reduced coenzymes took place. Consequently it was necessary to treat the alkaline extract as a mixture of both oxidized and reduced coenzymes (Table 3). When this was done, satisfactory recoveries of all the coenzymes were obtained after storage. Storage of the alkaline extract without neutralization reduces the degree of oxidation; but even with the shortest possible delay in estimation, no alkaline extract was found to contain only reduced coenzymes. In eight analyses of freshly prepared alkaline extracts, the mean values for the diphosphopyridine nucleotide and the triphosphopyridine nucleotide in the oxidized form were 31 and 18% respectively. Consequently, all alkaline extracts were analysed for both oxidized and reduced coenzymes and the sum of the values was taken as the original level of reduced coenzymes.

Initial values. The contents of the individual coenzymes in the livers of various animals are shown in Table 4.

The effect of starvation on the concentration and total content of coenzymes in rat livers was investigated. The weight of individual livers invariably fell with starvation. The concentration of coenzyme showed an initial increase to a higher level, which was either maintained or subsequently diminished, whereas the total quantity of coenzyme did not usually decrease.

Method of tissue preparation for use in metabolic experiments. The effect on the initial level of coenzyme and the suitability for metabolic studies are factors which must influence the choice of tissue preparation. No great differences were found between the levels of coenzymes in different preparations of a particular tissue (whole tissue, homogenate, mince and chopped tissue), though the values obtained with the last-mentioned were usually the lowest. In view of the lability of the coenzymes (see Tables 6 and 7) these low values are probably due to the length of time taken over this preparation. Further it was clear that portions of whole tissue could not be used in metabolic experiments. Accordingly, most of the subsequent work was done with either minces or homogenates.

Appreciable losses of coenzymes occurred in whole and minced liver kept at 0° (10–20% in 20 min.), though under similar conditions no

Table 3. *Behaviour of coenzymes during storage*

Rat-liver mince (1 g.; Fischer mince) was suspended in 14 ml. of medium with 25 mM-nicotinamide. The samples of extract not used for immediate analysis were frozen at −15°.

Coenzyme		Coenzyme content (μm-moles/g. fresh wt.) Analysed at once		Analysed after 24 hr.	
DPN		485		515	
TPN		103		115	
DPNH	as DPN	22	302	86	288
	as DPNH	280		202	
TPNH	as TPN	54	284	163	287
	as TPNH	230		124	

Table 4. *Coenzyme content of various tissues*

Figures in parentheses indicate the number of animals used. Values given are means ± S.E.M.; those marked with an asterisk were obtained from estimation of total oxidized and reduced coenzyme only.

Tissue	Preparation	Coenzyme content (μm-moles/g. fresh wt.) DPN	TPN	DPNH	TPNH	Total
Rat liver	Whole tissue (9)	—	—	—	—	899 ± 60*
Rat liver	Fischer mince (14)	—	—	—	—	895 ± 80*
Rat liver	Whole tissue (8)	485 ± 33	25 ± 6	153 ± 24	251 ± 24	912 ± 62
Rat liver	Fischer mince (14)	308 ± 41	64 ± 11	221 ± 38	261 ± 44	854 ± 81
Pigeon liver	Whole tissue (6)	596 ± 26	36 ± 17	93 ± 9	189 ± 36	924 ± 76
Pigeon liver	Fischer mince (5)	733 ± 53	82 ± 18	143 ± 60	288 ± 69	1246 ± 87
Mouse liver	Fischer mince (6)	513 ± 66	28 ± 7	137 ± 22	126 ± 12	801 ± 68
'Obese mouse' liver	Fischer mince (5)	434 ± 144	25 ± 3	162 ± 16	129 ± 12	750 ± 146

significant loss was seen in mince suspended in medium containing 0·05 M-nicotinamide. It follows that to obtain the most reliable initial values, any preparation should be carried to the stage of suspension in medium containing nicotinamide as speedily as possible. The relatively greater stability of the suspended-tissue preparation was clearly shown with pigeon liver, where the values obtained for whole tissue were significantly lower than for minced tissue (Table 4).

Measurement of coenzymes during incubation of various tissues. During the incubation of tissue preparations considerable destruction of coenzymes occurred (see Table 6). Three inhibitors of coenzyme-splitting enzymes were tested, namely nicotinamide, isoniazid and marsilid (see Zatman, Kaplan, Colowick & Ciotti, 1954). The last-named two substances markedly reduced the fluorescence obtained. When corrections for the interference were applied it was seen that the protective action of these compounds was not as effective as that of nicotinamide. When rat-liver mince was incubated at 38° for 20 min. 76% of the total coenzyme was destroyed. In the presence of nicotinamide (50 mM), isoniazid (5 mM) and marsilid (15 mM), the losses were 38, 56 and 96% respectively. The most satisfactory concentrations of nicotinamide were 25–50 mM, but even in the presence of this inhibitor and at 0° there was a considerable loss of coenzyme. The rate of destruction does not increase greatly as the temperature is raised from 25° to 37°.

Incubations of pigeon liver. Pigeon-liver homogenates were incubated in 0·01 M-lactate under aerobic and anaerobic conditions and with and without nicotinamide (Table 5). The effect of anaerobiosis was to increase the amount of TPNH found after incubation, though there was no marked difference in the levels of DPNH in oxygen or in nitrogen. The presence of nicotinamide increased the stability of the oxidized coenzymes, particularly DPN. The highest value for total coenzyme was found in oxygen and with nicotinamide, the lowest in oxygen and without nicotinamide. Nicotinamide also reduced the rate of endogenous respiration.

It was expected that in nitrogen the coenzymes would be predominantly in the reduced form, and this was so for the triphosphopyridine nucleotides; the constancy of the levels of DPNH in both oxygen and nitrogen may be due to the activity of DPNH pyrophosphatase of liver (see Jacobson & Kaplan, 1957). In oxygen, a marked increase in the proportion of oxidized coenzyme (notably DPN) was demonstrated only in the presence of nicotinamide, presumably because of the inhibition of diphosphopyridine nucleotidase activity. The values for total pyridine nucleotide are consistently high in the presence of nicotinamide because of a high proportion of the relatively stable TPNH.

Nicotinamide in pigeon-liver homogenates to which no substrate has been added has the effect of inhibiting the reduction of both TPN and DPN. In the presence of added lactate this effect is not found. These results are consistent with the existence of two distinct pathways for the reduction of pyridine nucleotides, one dependent on endogenous substrate (inhibited by nicotinamide) and the other on added lactate (not inhibited by nicotinamide).

Rat-liver incubations. When suspensions of rat-liver mince, final concentration 1 g. in 15 ml. of medium, with and without nicotinamide, were incubated at 37°, the results confirmed the marked protective effect of nicotinamide (Table 6). Measurements of the oxygen consumed by tissue in the two solutions showed that nicotinamide inhibited the respiration initially but maintained it at a higher level on prolonged incubation. It has been shown with pigeon-liver homogenates that nicotinamide in the absence of substrate inhibits

Table 5. *Coenzyme content of pigeon-liver homogenate found with and without addition of nicotinamide in the presence of lactate*

Pigeon-liver mince (1 g.) was suspended in 14 ml. of medium (Expt. 1) or of medium with 50 mM-nicotinamide (Expt. 2). All cups contained lactate (0·01 M). Incubations were carried out for 30 min. in Warburg vessels at 40°; the values given for Expts. 1 and 2 were obtained at the end of the incubation and are compared with initial levels of coenzyme in pigeon liver.

	Coenzyme content (μm-moles/g. fresh wt.)				
	Expt. 1		Expt. 2		
Coenzyme	O_2	N_2	O_2	N_2	Normal whole pigeon liver
DPN	238	202	346	223	584
TPN	51	44	65	21	14
DPN + TPN	289	246	411	244	598
DPNH	157	137	140	182	98
TPNH	36	225	120	213	149
DPNH + TPNH	193	362	260	395	247
Total	482	608	671	639	845

the reduction of coenzyme and also the oxygen consumption. The decrease in the level of reduced coenzymes in both tissues may account for the lower rate of endogenous respiration.

When suspensions of rat-liver mince were incubated in the presence and absence of pyruvate or lactate the only significant difference in the distribution of the coenzymes was a tendency for the level of DPNH to be maintained by the substrate (see Table 7). The effect produced by lactate on the distribution of coenzymes was not altered significantly by using a starved rat.

It was thought that as the proportion of undamaged cells in chopped tissue would be greater than in mince or homogenate, and that consequently the liberation of pyridine nucleotide-splitting enzymes into the tissue suspension would be reduced, a greater stability of coenzymes might be obtained with chopped tissue. To remove the enzymes released during chopping the preparation was washed twice with medium. However, no such increase in stability was achieved. Unlike the homogenate or mince the washed, chopped tissue comprises a suspension of mainly intact cells in a medium very low in substrate released from damaged cells. Consequently, a consistent difference observed was the extent to which the coenzymes became oxidized during incubation of chopped tissue, in contrast with homogenate and mince.

It was considered that chopped tissue offers a better possibility of observing a clear-cut effect of added substrate on the levels of oxidized and reduced coenzymes.

Sheep-heart incubations. The levels of oxidized and reduced coenzymes found after incubation of sheep-heart homogenates were virtually unaffected by the addition of pyruvate. The ability of sheep-heart homogenate to maintain the levels of reduced coenzymes was inferior to that of rat-liver mince whether or not pyruvate was added.

A similar effect to that which was found with washed chopped rat liver was also seen with the same preparation of sheep heart.

Table 6. *Effect of nicotinamide on the coenzyme level and respiration of rat-liver mince*

Rat-liver mince (1 g.) was suspended in 11 ml. of medium with or without 25 mM-nicotinamide. Incubations were carried out in Warburg vessels at 38° with air as the gas phase. Each vessel contained 3 ml. of suspension and 1 ml. of medium.

	Without nicotinamide				With nicotinamide			
	Coenzyme content (μm-moles/g. fresh wt.)			Increments of O_2 uptake	Coenzyme content (μm-moles/g. fresh wt.)			Increments of O_2 uptake
Time (min.)	Oxidized	Reduced	Total	(μl./g. fresh wt.)	Oxidized	Reduced	Total	(μl./g. fresh wt.)
0	310	320	630	—	635	455	1090	—
20	87	130	217	707	374	260	634	468
40	37	0	37	498	308	116	424	280
64	23	0	23	264	161	101	262	192
90	21	0	21	170	150	74	224	190
120	18	0	18	112	62	32	94	185
140	16	0	16	75	45	27	72	150

Table 7. *Coenzyme content of rat-liver mince incubated with and without pyruvate or lactate*

Rat-liver mince (1 g.) was suspended in 14 ml. of medium with 0·1 M-nicotinamide. The final concentration of pyruvate (Expt. 1) or of lactate (Expt. 2) was 10 mM. Incubations were carried out in Warburg vessels at 38° with air as the gas phase.

		Coenzyme content (μm-moles/g. fresh wt.)									
		DPN		TPN		DPNH		TPNH		Total	
Expt. no.	Time (min.)	No substrate	Pyruvate	No substrate	Pyruvate	No substrate	Pyruvate	No substrate	Pyruvate	No substrate	Pyruvate
1	0	346	302	112	150	148	181	158	196	764	829
	10	273	200	112	68	86	239	103	88	574	595
	17	145	180	100	61	92	149	43	112	480	502
2	0	293	329	60	57	89	90	128	106	570	582
	2	272	290	54	29	60	106	117	149	503	574
	8	228	265	35	56	81	96	115	137	459	554
	15	197	228	24	50	56	102	120	106	396	486
	30	132	134	0	18	7	91	101	103	240	346

DISCUSSION

Aspects of the method of estimation of coenzymes. The method for the analysis of pyridine nucleotides described above permits satisfactory estimation of DPN, TPN, DPNH and TPNH in concentrations as low as 1·5 μM. The sensitivity of this method is similar to that of Glock & McLean (1955) and Lowry *et al.* (1957), and ten times greater than that of Jacobson & Astrachan (1957). The method has two advantages over that of Lowry *et al.* (1957). First, the extraction procedure is improved. The method of Lowry *et al.* gives only 70% of the total nucleotide extractable in acid and alkaline medium, most of the deficit being in the reduced coenzymes. Secondly, the present method permits complete estimation of the extracted reduced nucleotides, even when these have been oxidized. The procedure of Lowry *et al.* (1957) results in underestimation of TPNH and overestimation of DPNH because it does not allow for this oxidation. An advantage over the method of Glock & McLean (1955) is the greater ease of preparation of the necessary enzymes. Numerous preparations of DPN–cytochrome *c* reductase made in this Laboratory for the procedure of Glock & McLean (1955) had only a small fraction of the required activity. Variation of the volume of the sample in which the fluorescence is developed may be made without major modification of the method, the upper limit of this volume being determined by the need for producing a concentration (6 N or greater) of sodium hydroxide during the development of the fluorescence. Sample volumes of 75 μl. have been used either when the concentration of total nucleotides or the relative concentration of one nucleotide was low. This variation was most frequently used to improve the reliability of endogenous TPN estimation.

It is essential to make frequent checks on the activity of the enzymes used in the method. Inactivity of either enzyme will not be detected by a discrepancy between the values for total nucleotides obtained (*a*) directly and (*b*) by addition of the values found for the individual species (e.g. Fig. 1: compare tube 1 with tube 2 plus tube 4). Other manipulative errors (e.g. failure to acidify a sample) will be detected by such a comparison.

Keto compounds present in the tissue extract were found to reduce the fluorescence, a finding compatible with the known ability of these compounds to quench fluorescence (Guinn, 1957; Lowry *et al.* 1957). During the extraction procedure substances which do not themselves quench fluorescence may be converted into compounds which do so interfere. For example, sucrose does not affect the estimation, but during the heating at low pH, fructose, which does interfere, is formed. Not only is the total amount of coenzyme underestimated but, because the alkaline treatment does not result in the same hydrolysis of sucrose, the estimation of the relative amounts of oxidized and reduced coenzymes is grossly in error.

It is clear that the rate of oxidation of the reduced nucleotides in the alkaline extracts adjusted to pH 8·0 is such that adequate allowance for this must be made in the analytical procedure. Lowry *et al.* have also noted an oxidation of DPNH in storage of pure solutions (i.e. other than tissue extracts), though the amount of DPN found was small. Singer & Kearney (1950) have shown that the rapid oxidation of reduced coenzymes in air is mediated by a number of free flavins, including riboflavin, which will be present in most tissue extracts. The main disadvantage of the modification described in this paper is the extent to which it extends the manipulations involved. Unsuccessful attempts were made either to prevent the oxidation by the addition of a number of anti-oxidants and reducing compounds, or to complete it by adding riboflavin or by shaking the sample in an atmosphere of oxygen.

Coenzyme content of animal tissues. The values presented in this paper for the levels of coenzymes in rat liver agree fairly closely with those of Glock & McLean (who used a method in which oxidation during storage cannot result in the underestimation of reduced nucleotides), but differ, especially in the amount of TPNH, from those of Lowry *et al.* (1957). This difference is probably related to the modifications of the methods of extraction and estimation. The magnitudes of the standard errors of the mean given in Table 5 are greater than those presented by Glock & McLean (1955). Values for the total coenzyme level of rat-liver mince as high as 1400 μm-moles/g. fresh wt. and as low as 600 μm-moles/g. fresh wt. have been observed. With rat liver, it has been shown that damage to the tissue lowers the level of coenzymes and the initial values obtained in experiments necessitating longer handling of tissue preparations before extraction were also somewhat lower. The levels of coenzymes found in extracts of solid pieces of pigeon liver were significantly lower than those found in minces of the same tissue. It seems possible that partial inactivation of coenzyme-splitting enzymes may take place when the tissue is minced and suspended in buffer at pH 7·4, since Jacobson & Kaplan (1957) have shown that these enzymes are inactive at this pH; removal of connective tissue and blood vessels during mincing may also increase the apparent concentration of the coenzymes in minces. The initial amount of reduced coenzyme in minced tissue was greater than that of comparable whole-tissue preparations, and this difference may reflect the ease with which

endogenous substrate and the coenzymes interact when cells are disrupted.

Measurement of coenzymes during the incubation of tissue preparations. The rapid loss of coenzyme during the incubation of the various tissue preparations makes it difficult to examine the factors controlling the coenzyme level. The addition of nicotinamide reduced the loss but did not prevent it. However, nothing more effective has been found. Nicotinamide does not only protect the coenzymes from destruction but also reduces the initial rate of endogenous respiration and prevents the reduction of coenzyme by endogenous substrate, an effect also noted in unpublished experiments with mitochondria. It is advisable therefore to keep the concentration of nicotinamide at a minimum. A comparison of initial values for the oxidized and reduced coenzymes of rat-liver minces prepared with and without nicotinamide suggests that the oxidized coenzymes are more rapidly destroyed, and this preferential destruction means that the observed ratios of oxidized and reduced coenzyme are altered in a way which may not be paralleled in the intact animal. The addition of substrates producing a greater proportion of reduced coenzyme may also help to maintain the total coenzyme level. A precise interpretation of all such experiments is difficult and only clear-cut shifts in ratios can be taken as having a significant relationship to the effect of the metabolites added in the experiments. Results are more meaningful when the time of incubation is short.

Changes in the levels of oxidized and reduced coenzyme during short periods of incubation of homogenates and minces of rat liver, pigeon liver and sheep-heart muscle were not influenced greatly by the addition of substrate. Sometimes, with longer periods of incubation, most of the reduced coenzymes were removed in the absence of added substrate, whereas with lactate and pyruvate the disappearance of TPNH and DPNH was greatly diminished. However, there was always a considerable loss of total coenzyme. It was possible to demonstrate more rapid and clear-cut effects of added substrate with washed, chopped tissue, but the total coenzyme level in these preparations was always low. Thus as short times of incubation with added substrate did not affect the ratios of oxidized and reduced coenzymes, and as longer incubations or the use of washed tissue resulted in considerable loss of total coenzymes, it was impossible to correlate the ratios of oxidized and reduced coenzymes with the influence of added substrate. None of the preparations used seems to be suited for investigating the influence of levels of pyridine nucleotides on metabolic reactions. Accordingly, the investigations are being pursued with mitochondrial preparations.

SUMMARY

1. A fluorimetric method for the estimation of di- and tri-phosphopyridine nucleotide and reduced di- and tri-phosphopyridine nucleotide in animal tissues is described. Acid and alkaline extractions of the tissues are used for the oxidized and reduced coenzymes respectively. After extraction, the four species of coenzymes are separated by treatment with specific enzymes and selective destruction of oxidized and reduced forms with acid and alkali. The fluorescence of the separated coenzymes is developed by heating with 6 N-sodium hydroxide.

2. The method can be used for the estimation of pyridine nucleotides in concentrations as low as 1·5 μM, and quantities as small as $0{\cdot}1 \times 10^{-9}$ mole have been determined.

3. Conditions affecting the accuracy and specificity of the method have been investigated.

4. Values for the concentrations of the coenzymes in various tissues immediately after death are presented (see Table 5).

5. The suitability of a number of tissue preparations for use in metabolic experiments involving incubation and the subsequent estimation of the coenzymes has been investigated.

6. There is a rapid destruction of coenzymes on incubation of homogenates and minced or chopped tissues. Pyruvate and lactate usually decreased the rate of destruction and in some experiments maintained a high level of reduced coenzymes.

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Metal-Catalysed Auto-oxidation of Isoniazid

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Isoniazid (*iso*nicotinic acid hydrazide) is stable in the solid state and in concentrated aqueous solutions. In dilute aqueous solutions, however, it breaks down under certain conditions. This breakdown has been shown to require oxygen (and hence may be described as an auto-oxidation) and certain metal ions, either free, such as Mn^{2+}, Cu^{2+}, Fe^{3+}, or combined as haemin; and to be accelerated by increase of pH (Pansy, Koerber, Stander & Donovick, 1953; Bönicke, 1954; Barry, Conalty, Denneny, Gaffney & Winder, 1954; Lewin & Hirsch, 1955; Krüger-Thiemer, 1955; Albert & Rees, 1955).

The breakdown catalysed by haemin has been studied in some detail (Krüger-Thiemer, 1955; Albert & Rees, 1955). The work in the present paper was designed to give further information on the auto-oxidation catalysed by Mn^{2+} and Cu^{2+} ions, both because of its importance in biological and biochemical studies with isoniazid and because these model systems throw some light on the metal-catalysed auto-oxidation of other biologically active substances.

EXPERIMENTAL

Determination of rate of isoniazid breakdown. The required solutions (buffer, metal-salt solutions, chelating agents, peroxidase etc.) were pipetted into 12 cm. × 1·5 cm. Pyrex test tubes with sufficient water to bring the total volume to 3 ml. Each treatment was usually set up in duplicate. A portion (1 ml.) of a solution of isoniazid or other hydrazide was then added, the tubes were shaken, 1 ml. samples were taken immediately for determination of isoniazid and the tubes incubated in a water bath at the required temperature. After the appropriate period the tubes were removed, rapidly cooled to room temperature and further 1 ml. samples taken for determination of isoniazid.

Velocity constants of isoniazid breakdown were calculated from the formula for a first-order reaction

$$k = \frac{2{\cdot}3}{t} \log \frac{c_0}{c_t} \text{ (hr.}^{-1}\text{)},$$

where c_0 is the initial concentration of isoniazid and c_t is the concentration of isoniazid after t hr. These constants were normally calculated for a single reaction time (15 or 30 min. for rapid reactions, 60 or 120 min. when the reaction was slower). Isoniazid breakdown deviated considerably from a first-order reaction under many experimental conditions, but in the early stages of the reaction the deviation was not great (Table 5), and these velocity constants were sufficiently accurate for the purpose of the comparisons made in this paper.

Estimation of isoniazid. Isoniazid solution (1 ml., containing 0·2 μmole or less), 3 ml. of 2 N-H_2SO_4 and 1 ml. of reagent (0·6 g. of dimethylaminobenzaldehyde in 20 ml. of ethanol plus 2 ml. of 10 N-HCl) were pipetted into 12 cm. × 1·5 cm. Pyrex test tubes, which were capped with bubble-caps and placed in a boiling-water bath for 45 min. The solutions were cooled, made up to 6 ml. and the extinctions were read at 450 mμ. Appropriate blank and standard determinations were carried out.

This method estimates hydrazine freed by hydrolysis, and hence estimates not only isoniazid but di*iso*nicotinylhydrazine also. The latter gives 20% of the extinction given by an equimolecular amount of isoniazid.

Estimation of hydrogen peroxide. Hydrogen peroxide was estimated by a titanium sulphate method (Winder, 1956).

Products of isoniazid auto-oxidation. Isoniazid (20–200 mM) was shaken with 5 mM-$CuSO_4$ in 20 mM-borate buffer, pH 7·5, or with 5 mM-$MnCl_2$ in 10 mM-phosphate buffer, pH 7·5, in an open conical flask in a water bath at 37° for about 6 hr., the pH being readjusted to 7·5 at intervals with 5 N-NaOH. Samples of the solutions were then spotted on Whatman no. 1 paper and developed by the ascending technique for 18 hr. at room temperature in propan-2-ol–water (80:20) or in the butanol phase of butanol–acetic acid–water (50:10:40). Isoniazid, *iso*nicotinic acid, di*iso*nicotinyl hydrazine and *iso*nicotinamide were identified by their R_F values compared with the authentic substances run as markers, and by the use of the spray reagents of Cuthbertson, Ireland & Wolff (1953). *iso*Nicotinamide was eluted from larger-scale chromatograms and its absorption spectrum in 95% ethanol and in water compared with those of synthetic material. Di*iso*nicotinylhydrazine showed an orange fluorescence on excitation with ultraviolet light of λ 366 mμ and gave a pale yellow with cyanogen bromide and benzidine. These helped to distinguish this substance from *iso*nicotinamide, with which its spot on the chromatogram partly overlapped.

Polymerization of styrene. Borate buffer (4 ml.; 0·2 M; pH 8·0), 4 ml. of 20 mM-$CuSO_4$, 4 ml. of styrene and 4 ml. of 100 mM-isoniazid were pipetted into a 100 ml. conical flask, which was then plugged with cotton wool and shaken at 37° for 3 hr. Similar mixtures, but with the copper or the isoniazid omitted, were also shaken. After incubation, 1 ml. of conc. HCl was added to each flask to dissolve any precipitated copper salts. The contents of the flasks were well shaken, transferred to centrifuge tubes and centrifuged at 3000 *g* for 10 min. A thin layer of white polymer-coated styrene droplets was observed between the water and styrene layers from the flask containing isoniazid and copper, but not from the flasks containing isoniazid or

copper alone. Because of difficulties introduced by evaporation of the styrene, satisfactory quantitative estimates of the amount of polymer formed were not obtained.

Effect of isoniazid auto-oxidation on aromatic compounds. Borate buffer (1 ml.; 0·1 M; pH 7·5) 1 ml. of 10 mM-substrate, 1 ml. of mM-isoniazid and 1 ml. of 0·2 mM-$CuSO_4$ were pipetted into a 25 ml. conical flask, which was shaken at 37°. Similar mixtures without isoniazid were also incubated. Absorption curves were obtained after various intervals. Substrates used were phenol, cresol, catechol and aniline. Benzene was also used as a substrate, but with ten times the above-mentioned concentrations of isoniazid and copper.

Materials. *iso*Nicotinamide and di*iso*nicotinylhydrazine were prepared by Dr Peter Mitchell, of these Laboratories, by the methods of Ainsworth (1954) and Albert & Rees (1955) respectively. The hydrazides of acids other than *iso*nicotinic acid were also gifts from Dr Mitchell. Isoniazid was obtained from the Nepera Chemical Co., and *iso*nicotinic acid from L. Light and Co. Ltd.

Peroxidase (horse-radish, P.Z. 170) and catalase (ox-liver, crude) were purchased from Nutritional Biochemicals Corp. Haemin was obtained from L. Light and Co. Ltd.

Styrene (L. Light and Co. Ltd.) was washed with water and distilled. Benzene was purified by shaking with conc. H_2SO_4 for several hours, leaving with metallic sodium for 18 hr. and distilling.

The other chemicals used were of A.R. quality. Manganese was used as $MnCl_2$, copper as $CuSO_4$ and iron as $FeCl_3$.

No special precautions were taken to eliminate trace metals. Glass-distilled water was used. Glassware was cleaned in chromic acid and thoroughly rinsed in tap water and distilled water.

Buffers were prepared by dissolving potassium dihydrogen phosphate, sodium bicarbonate, boric acid, sodium diethylbarbiturate, glycine, sodium citrate or 2-amino-2-hydroxymethylpropane-1:3-diol (tris) in water, adjusting to the required pH with 10 N-NaOH or 10 N-HCl, making up to the required volume and re-checking the pH.

All pH adjustments were made with a Radiometer pH meter 21, and extinction readings were made with a Beckman model DU spectrophotometer.

RESULTS

Table 1 shows the effect of trace amounts of Mn^{2+}, Cu^{2+}, Fe^{3+} ions and haemin on the auto-oxidation of isoniazid in various buffers, pH 7·5, at 37°. Slow and variable oxidation took place in some of the buffers in the absence of added metals, which was presumably due to traces of metals in the solutions, and could be completely eliminated by the use of chelating agents. The catalytic effect of Cu^{2+} and Mn^{2+} ions was strong in some buffers and almost absent in others. The effect of Mn^{2+} ions was detectable at a concentration as low as 0·05 μM in 25 mM-phosphate buffer.

There was considerable variation in oxidation rates under apparently similar conditions from experiment to experiment, as may be seen by comparing the results in the different tables, but agreement between replicate determinations in the same experiment was good, and the pattern of results within an experiment was always reproducible. The causes of inter-experimental variation have not been discovered.

Manganese dioxide and Cu^{2+} ion oxidized isoniazid in the absence of oxygen in phosphate buffer, pH 7·5, at 37°, becoming reduced to Mn^{2+} and Cu^{+} ions. This may therefore be assumed to take place in the auto-oxidation of dilute solutions of isoniazid in the presence of traces of these metals. Cu^{+} ion is reoxidized by oxygen to Cu^{2+}, explaining the catalytic effect. On the other hand, Mn^{2+} ion is not appreciably oxidized to MnO_2 by oxygen under the above conditions. Small traces of MnO_2 may, however, be formed and these may be sufficient to explain the catalytic effect. It is possible, on the other hand, that oxygen reacts with a metal–isoniazid chelate, and that oxidation of the isoniazid takes place by electron transfer within the complex without a change in valency of the free metal ions.

Co^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Al^{3+} and molybdate ions had no appreciable effect on isoniazid in phosphate buffer, pH 7·5, at 37°.

Effect of pH. The rate of auto-oxidation of isoniazid in the presence of Cu^{2+} or Mn^{2+} ions in 25 mM-phosphate buffer was increased by raising

Table 1. *Effect of metals on the velocity constant of the auto-oxidation of isoniazid in various buffers*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Isoniazid was initially present at 0·2 mM, and the buffers and metals were as given in the table. pH was 7·5. Isoniazid was determined in samples taken at 0 and 120 min. when haemin was present, and at 0 and 30 min. in the other cases, and the first-order velocity constant of its disappearance was calculated.

	Metal catalyst			
Buffer	0·045 mM-Mn^{2+} k (hr.$^{-1}$)	0·045 mM-Cu^{2+} k (hr.$^{-1}$)	0·045 mM-Fe^{3+} k (hr.$^{-1}$)	0·045 mM-Haemin k (hr.$^{-1}$)
25 mM-Phosphate	1·2	0·29	0·01	0·46
5 mM-Phosphate	1·8	0·06	0·06	0·43
25 mM-Bicarbonate	2·9	1·7	0·10	0·57
25 mM-Borate	0·03	3·4	0·06	0·43
5 mM-Veronal	0·00	2·5	0·03	0·29
25 mM-Glycine	0·09	0·00	0·05	0·31
25 mM-Citrate	0·02	0·00	—	0·44
25 mM-Tris	0·09	0·02	—	0·26

the pH over the range studied (Table 2). Oxidation by Mn^{2+} ion was more strongly affected by pH changes than oxidation by Cu^{2+} ion.

Effect of temperature. Over the range 30–40° a 5° increase in temperature resulted in a 1·6-fold increase in rate of auto-oxidation of isoniazid, whether this was catalysed by Cu^{2+} or Mn^{2+} ions.

Effect of chelating agents. The effects of a number of chelating agents on the Mn^{2+}-catalysed auto-oxidation of isoniazid are shown in Table 3. Ethylenediaminetetra-acetate, diethyldithiocarbamate and αα′-dipyridyl inhibited the oxidation when present at concentrations higher than equimolar to that of the Mn^{2+} ions, but they stimulated when present at lower concentrations. These facts can be explained most simply on the assumption that formation of 1:1 complexes with these agents increased the effectiveness of Mn^{2+} ion as a catalyst under the conditions of this experiment, whereas the formation of 1:2 complexes inactivated the Mn^{2+} ion.

o-Phenanthroline and 8-hydroxyquinoline showed rather different behaviour. The former inhibited the auto-oxidation at a molar concentration one-tenth of that of the Mn^{2+}; the latter did not inhibit until present at a concentration more than twice that of the Mn^{2+} ion.

Effect of hydrogen peroxide. Hydrogen peroxide markedly inhibited the auto-oxidation catalysed by Mn^{2+} ion (Table 4). Presumably the hydrogen peroxide reduced the oxidized form of the catalyst (MnO_2 or its equivalent), so that less was available for the oxidation of isoniazid. Conversely, catalase stimulated this auto-oxidation by removing hydrogen peroxide, which was normally a reaction product.

On the other hand, hydrogen peroxide strongly stimulated auto-oxidation of isoniazid catalysed by Cu^{2+} ion. In this case hydrogen peroxide did not react with the oxidized form of the catalyst, but presumably oxidized the reduced form and thereby made more Cu^{2+} ion available for isoniazid oxidation. The reaction of Cu^{+} ion with hydrogen peroxide probably produced free OH radicals (Haber & Weiss, 1934; Baxendale, Evans & Park, 1946; Waters, 1945), which may also oxidize isoniazid.

Effect of concentration of isoniazid. The auto-oxidation of isoniazid catalysed by Mn^{2+} or Cu^{2+} ions followed a first-order course under certain conditions, and hence unimolecular velocity 'constants' have been calculated throughout this paper. However, the 'constant' when Mn^{2+} ion was catalyst in fact decreased during the course of the reaction, particularly towards the end of the reaction, and with lower concentrations of isoniazid (Table 5). One cause of the decrease of the reaction 'constant' during the reaction was the

Table 2. *Effect of* pH *on the velocity constant of the auto-oxidation of isoniazid in phosphate buffer*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0·2 mM; phosphate, 25 mM. Concentration of the metal ions are given in the table. Isoniazid was determined in samples taken at 0 and 30 min. and the first-order velocity constant of its disappearance was calculated.

pH	No metal catalyst k ($hr.^{-1}$)	0·045 mM-Cu^{2+} k ($hr.^{-1}$)	0·045 mM-Mn^{2+} k ($hr.^{-1}$)
7·5	0·005	0·18	1·2
7·0	0·002	0·14	0·60
6·5	0·000	0·11	0·18
6·0	0·000	0·05	0·11
5·5	0·000	0·02	0·002

Table 3. *Effect of chelating agents on the velocity constant of auto-oxidation of isoniazid in phosphate buffer catalysed by* Mn^{2+} *ion*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0·2 mM; Mn^{2+}, 0·045 mM; phosphate, 25 mM; pH 7·5. Isoniazid was determined in samples taken at 0 and 30 min., and the first-order velocity constant of its disappearance was calculated.

Additions	k ($hr.^{-1}$)
Control	1·08
0·003 mM-Ethylenediaminetetra-acetate	2·18
0·09 mM-Ethylenediaminetetra-acetate	0·05
0·008 mM-Diethyldithiocarbamate	1·96
0·09 mM-Diethyldithiocarbamate	0·06
0·008 mM-αα′-Dipyridyl	1·75
0·09 mM-αα′-Dipyridyl	0·06
0·006 mM-*o*-Phenanthroline	0·74
0·09 mM-*o*-Phenanthroline	0·62
0·017 mM-8-Hydroxyquinoline	2·28
0·09 mM-8-Hydroxyquinoline	1·77
0·20 mM-8-Hydroxyquinoline	0·12

Table 4. *Effect of hydrogen peroxide and of catalase on the velocity constant of isoniazid auto-oxidation in phosphate buffer*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0·2 mM; Cu^{2+}, 0·045 mM; Mn^{2+}, 0·045 mM; phosphate, 25 mM; H_2O_2, 0·37 mM; crude catalase, 12·5 μg./ml.; pH 7·5. Samples were taken at 0 and 15 min., and the first-order velocity constant of isoniazid disappearance was calculated.

Additions	k ($hr.^{-1}$)
Cu^{2+}	0·21
$Cu^{2+} + H_2O_2$	3·00
Cu^{2+} + catalase	0·12
Mn^{2+}	2·42
$Mn^{2+} + H_2O_2$	0·24
Mn^{2+} + catalase	3·86
H_2O_2	0·04
Catalase	0·00

accumulation of hydrogen peroxide, which inhibited the Mn^{2+}-catalysed auto-oxidation. In addition, the metal ions may become converted into an inactive form.

When the initial concentration of isoniazid was increased (with constant concentration of metal ion), the amount of isoniazid destroyed in a short time increased also, though not exactly proportionately to the initial concentration of isoniazid (Table 6). Up to a certain concentration of isoniazid (which depended on the metal catalyst and on the complexing agent present) the amount destroyed increased slightly more rapidly than the initial concentration. Above this concentration the amount destroyed increased considerably more slowly. These results suggest that at low concentrations of isoniazid the metal ions do not attain their full activity, and that at high concentrations of isoniazid the metal-ion catalyst tends towards 'saturation' and the reaction towards zero order. This last-named effect may be due to the rate of re-oxidation of the metal ions limiting the rate of the reaction, or to formation of a more stable chelate when the ratio of isoniazid to metal is high.

Products of auto-oxidation of isoniazid. The auto-oxidation products were studied in solutions more concentrated than those used for the other studies, and hence the results may not be exactly comparable. The previously recorded products of isoniazid auto-oxidation, *iso*nicotinic acid and di*iso*nicotinyl hydrazine (Krüger-Thiemer, 1955; Albert & Rees 1955) were found when either Mn^{2+} or Cu^{2+} ion was the catalyst. However, particularly with Mn^{2+} ion as catalyst, substantial quantities of a third substance were found. This was identified as *iso*nicotinamide by chromatography against synthetic *iso*nicotinamide in propan-2-ol–water and in butanol–acetic acid–water, by the blue colour of its spot with cyanogen bromide and benzidine and by its absorption spectrum after elution from chromatograms (Table 7).

The formation of *iso*nicotinamide was presumably due to reduction of isoniazid or an intermediate oxidation product [perhaps the radical 4-*iso*nicotinamido ($4\text{-}NC_5H_4\cdot CO\cdot NH^{\bullet}$) suggested by Albert & Rees (1955)] by the reduced form of the metal-ion catalyst.

During isoniazed auto-oxidation catalysed by Mn^{2+} ion, hydrogen peroxide accumulated in amounts rather more than equimolar to those of the isoniazid destroyed. The reactions involved in the auto-oxidation may be assumed to be something as follows:

Table 5. *Change in the first-order velocity constant during the course of the auto-oxidation of isoniazid in phosphate buffer catalysed by* Mn^{2+} *ion*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: Mn^{2+}, 0·045 mM; phosphate, 25 mM; isoniazid as indicated in the table; pH 7·5. Isoniazid was determined in samples taken at 0 min. and at the indicated times, and first-order velocity constants of isoniazid disappearance were calculated for each successive interval, i.e. 0–15 min.; 15–30 min., etc.

Time from start of reaction (min.)	Initial concn. of isoniazid	
	1·8 mM	0·18 mM
	k ($hr.^{-1}$)	k ($hr.^{-1}$)
15	0·27	2·3
30	0·43	1·6
60	0·40	1·2
120	0·32	0·54
240	0·22	0·33

Table 6. *Effect of the concentration of isoniazid on its auto-oxidation in phosphate buffer*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: Mn^{2+}, 0·045 mM; Cu^{2+}, 0·045 mM; ethylenediaminetetra-acetate (EDTA), 0·003 mM; phosphate, 25 mM; pH 7·5.

	Catalyst		
	Mn^{2+}	Mn^{2+} + EDTA	Cu^{2+}
	Incubation period (min.)		
Initial concn. of isoniazid (μmoles/l.)	30	30	60
	Isoniazid destroyed (μmoles/l.)		
18·2	—	15·3	1·8
45·6	17·2	39·4	5·4
91·3	36·5	77·4	12·1
182	88·4	136	30·9
365	169	—	80·1
913	192	—	136

Table 7. *Absorption maxima of* iso*nicotinamide eluted from the chromatogram*

	Absorption maximum (mμ)	
	95% Ethanol	Water
Isoniazid	264	263
*iso*Nicotinic acid	272	263
Di*iso*nicotinylhydrazine	263	266
*iso*Nicotinamide	270	268
'*iso*Nicotinamide' from auto-oxidation	270	268

$$Mn^{2+} + O_2 + 2H_2O = MnO_2 + H_2O_2 + 2H^+ \quad (1)$$

$$2(4\text{-}NC_5H_4\cdot CO\cdot NH\cdot NH_2) + 2MnO_2 = 2(4\text{-}NC_5H_4\cdot CO\cdot NH^{\bullet}) + 2Mn^{2+} + 4OH^- + N_2 \quad (2)$$

$$2(4\text{-}NC_5H_4\cdot CO\cdot NH^{\bullet}) = 4\text{-}NC_5H_4\cdot CO\cdot NH\cdot CO\cdot H_4C_5N(4\text{-}) \quad (3)$$

$$2(4\text{-}NC_5H_4\cdot CO\cdot NH^{\bullet}) + MnO_2 + 2H_2O = 2(4\text{-}NC_5H_4\cdot CO_2H) + Mn^{2+} + 2OH^- + N_2 \quad (4)$$

$$2(4\text{-}NC_5H_4\cdot CO\cdot NH^{\bullet}) + Mn^{2+} + 2H_2O = 2(4\text{-}NC_5H_4\cdot CO\cdot NH_2) + MnO_2 + 2H^+ \quad (5)$$

The exact amount of hydrogen peroxide formed thus depends on the amounts of the different breakdown products of isoniazid. Hydrogen peroxide accumulated in this system because Mn^{2+} ion did not reduce hydrogen peroxide under the conditions of our experiments, though under other conditions Mn^{2+} ion can transfer single electrons to hydrogen peroxide to form free OH radicals (Baxendale *et al.* 1946; Waters, 1945).

Peroxide did not accumulate when Cu^{2+} ion was the auto-oxidation catalyst. It has been mentioned already that Cu^{2+} ion probably accepts single electrons from hydrogen peroxide to form free OH radicals in this system, as has been shown to take place under other conditions (Baxendale *et al.* 1946; Waters, 1945). It is probable that the reoxidation of Cu^{+} by oxygen takes place by a succession of single electron transfers with successive formation of $HO_2^{\cdot}$, H_2O_2, $HO^{\cdot}$ and water (Weiss, 1958). The formation of free radicals during the Cu^{2+}-catalysed auto-oxidation of isoniazid is supported by the fact that this system stimulates polymerization of styrene. Further, the isoniazid–Cu^{2+}–oxygen system gave coloured reaction products on incubation with benzene, aniline and monohydroxy- and dihydroxy-phenols, whereas the Cu^{2+}–oxygen system produced much less colour or none. This suggests formation of OH radicals, which attacked the aromatic rings, but the presence of isoniazid in the system introduced a certain ambiguity into the results. Short-lived reaction intermediates, such as the free radicals $4\text{-}NC_5H_4\cdot CO\cdot NH^{\cdot}$ or $4\text{-}NC_5H_4\cdot CO^{\cdot}$, may be formed from isoniazid itself and these may be responsible for some of the effects. This ambiguity can be removed only by further characterization of the reaction products. The isoniazid–Cu^{2+}–oxygen system also irreversibly decolorized certain dyes, e.g. methylene blue, Evans blue and crystal violet.

Effect of peroxidase on isoniazid. It has been claimed that peroxidase oxidizes isoniazid (Krüger-Thiemer, 1958; Tirunarayanan & Vischer, 1959). The results in Table 8, however, show that peroxidase had little effect on isoniazid, even when present at very high concentrations. Old solutions of peroxidase had more effect, which may be due to the liberation of some free haem derivatives from the peroxidase, and the results of the abovementioned workers may be explicable along these lines.

When hydrogen peroxide or phenol was added to the peroxidase–isoniazid system little effect on the isoniazid was observed, but when both were added together the isoniazid was rapidly destroyed. Hence it would appear that peroxidase does not oxidize isoniazid to *iso*nicotinic acid, as has been suggested (Krüger-Thiemer, 1958), but that quinone intermediates in the oxidation of phenols by peroxidase react with isoniazid. For example, when peroxidase, quinol, hydrogen peroxide and isoniazid were incubated together a dark-brown insoluble product resulted, similar to that obtained when isoniazid was incubated under the same conditions with *p*-quinone. The product of both reactions on chromatography in propan-2-ol–water gave the same spot, with R_F 0·88, which gave a pink some hours after exposure to cyanogen bromide and benzidine.

Peroxidase plus phenol increased the rate of oxidation of isoniazid by Mn^{2+} ion, presumably because the hydrogen peroxide formed in the Mn^{2+}-catalysed oxidation was used by the peroxidase–phenol system for the removal of further isoniazid. The reaction rate is apparently increased four times, but first-order velocity constants give a false picture in this case. The figures for the amount of isoniazid destroyed per unit time are in keeping with the formation of rather more than one molecule of hydrogen peroxide for each molecule of isoniazid auto-oxidized, and the use of each molecule of hydrogen peroxide for the destruction of a further molecule of isoniazid by peroxidase–phenol.

Hydrogen peroxide formed in this way is more rapid and efficient in the destruction of isoniazid than is hydrogen peroxide added in bulk. Addition of hydrogen peroxide actually slows down the destruction of isoniazid by Mn^{2+}–peroxidase–phenol. Excess of hydrogen peroxide either partially inhibited the peroxidase action, or changed the nature or amount of the quinone

Table 8. *Effect of peroxidase on isoniazid*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0·2 mM; phosphate, 25 mM; H_2O_2, 0·4 mM; phenol, 0·5 mM; Mn^{2+}, 0·045 mM; NaN_3, 0·4 mM; peroxidase, 5 μg./ml. except where specified otherwise; crude catalase, 25 μg./ml.; pH 7·5. Isoniazid was determined in samples taken at 0 and 15 min., and first-order velocity constants of isoniazid disappearance were calculated.

Additions	k (hr.$^{-1}$)
Control	0
Peroxidase	0
Phenol	0
Peroxidase + phenol	0·15
Peroxidase + H_2O_2	0·10
Peroxidase + phenol + H_2O_2	3·0
Peroxidase + phenol + H_2O_2 + NaN_3	3·9
Peroxidase + phenol + H_2O_2 + catalase	2·0
Mn^{2+}	2·6
Mn^{2+} + peroxidase + phenol	10·0
Mn^{2+} + peroxidase + phenol + NaN_3	14·0
Mn^{2+} + peroxidase + phenol + catalase	12·0
Peroxidase (50 μg./ml.)	0·06
Peroxidase (50 μg./ml.; stored for 4 months in solution)	0·20

Table 9. *Comparison of the velocity constants of the auto-oxidation of hydrazides with their growth-inhibitory activity for* Mycobacterium tuberculosis

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. The hydrazides were initially present at 0·2 mM. pH 7·5. Samples were taken at 0 and 120 min. for haemin, at 0 and 30 min. for the others, and the first-order velocity constants of the disappearance of the hydrazides were calculated. (*a*) Bernstein, Lott, Steinberg & Yale (1952) and Bernstein *et al.* (1953): Ravenel strain in modified Kirchner medium. (*b*) R. Knox (Albert, 1956): H37Rv strain in semi-solid Dubos medium. (*c*) Winder & Denneny: BCG strain in Proskauer & Beck medium with 2·5% of serum.

Hydrazide of	0·045 mM-Mn^{2+} 25 mM-phosphate k ($hr.^{-1}$)	0·045 mM-Cu^{2+} 5 mM-veronal k ($hr.^{-1}$)	0·01 mM-Haemin 25 mM-phosphate k ($hr.^{-1}$)	Minimum inhibitory concn. for *M. tuberculosis* (μg./ml.) (*a*)	(*b*)	(*c*)
*iso*Nicotinic acid	1·5	2·4	0·013	0·02	0·025	0·03
Picolinic acid	—	2·2	0·079	0·15	1·5	1·5
Nicotinic acid	—	3·0	0·011	2·0	25·0	>10·0
Furoic acid	—	3·5	0·036	0·06	—	1·0
Thiophencarboxylic acid	2·2	9·0	0·022	8·0	—	1·0
Benzoic acid	1·6	4·6	0·092	3·0	12·5	>100
o-Chlorobenzoic acid	—	2·5	0·005	—	—	>100
p-Hydroxybenzoic acid	0·25	15·5	0·18	12·0	—	—
p-Aminobenzoic acid	—	6·9	0·13	6·0	—	—
Pyrrolidonecarboxylic acid	0·53	1·9	0·009	—	—	>100
Maleic acid	—	10·0	0·31	—	—	—
Cyanacetic acid	1·0	1·0	0·23	—	3·0	>100

products formed. The stimulation by subinhibitory levels of azide of destruction of isoniazid by these systems also requires explanation. These levels of azide may modify the products of peroxidase action on phenols.

Catalase inhibited the destruction of isoniazid by peroxidase–phenol–peroxide, for obvious reasons. However, it increased the destructive effect of peroxidase–phenol–Mn^{2+}.

Auto-oxidation of some other hydrazides. The auto-oxidation of a number of other hydrazides was also studied. Although all of those investigated were auto-oxidized, the rates varied considerably, as did the effects of the different catalysts (Table 9). The series was too limited to permit generalizations on the effect of structure on the rate of auto-oxidation. No correlation was observed between activity *in vitro* against *Mycobacterium tuberculosis* and auto-oxidation rate.

DISCUSSION

The observations in this paper show that isoniazid and other hydrazides may be quite rapidly auto-oxidized under conditions prevailing in bacterial and enzyme systems. Thus at pH 7·5 and 37°, with 2 μg. of Mn^{2+} ion/ml. present, isoniazid (100 μg./ml.) may be reduced to half its concentration in less than 30 min. The oxidation rate is governed by the metal ions present, the pH and the anions present, and may also be affected by chelating agents and by hydrogen peroxide. Hence, studies on the effect of isoniazid on bacterial and biochemical systems should be accompanied by some examination of the rate of breakdown of isoniazid under the exact conditions used, both because it will affect the concentration of isoniazid present and because the products of auto-oxidation may be responsible for some of the effects attributed to isoniazid itself.

Suggestions have been made that breakdown of isoniazid is involved in its action on *Mycobacterium tuberculosis* (Albert & Rees, 1955; Winder, 1956, 1959; Krüger-Thiemer, 1958). However, the evidence in favour of these hypotheses is indirect, and there are a number of difficulties in their way. First, no correlation has been found between the auto-oxidation rates of hydrazides and their activity against *M. tuberculosis in vitro*, so that other properties of the molecules must be assumed to play a large part in determining their activity. Secondly, the stable products of isoniazid auto-oxidation (*iso*nicotinic acid, di*iso*nicotinyl hydrazine, *iso*nicotinamide and hydrogen peroxide) are much less toxic for *M. tuberculosis* than is isoniazid, although, as has been pointed out (Winder, 1956; Krüger-Thiemer, 1958), their toxicity might be considerably greater if they are formed within the mycobacterial cell.

However, in addition to the above-mentioned products, the isoniazid–Cu^{2+}–oxygen and isoniazid–haemin–oxygen systems form free radicals under suitable conditions. These radicals may be of two types: radicals derived from the reduction of oxygen, such as $HO^{\cdot}$, and the reaction intermediates derived from the oxidation of isoniazid, which may be short-lived free radicals such as

4-$NC_5H_4 \cdot CO \cdot NH^{\cdot}$ or 4-$NC_5H_4 \cdot CO^{\cdot}$. The former type of radicals show oxidizing and hydroxylating activity and the latter would be expected to attack certain chemical groupings. Therefore, in the presence of isoniazid and oxygen, Cu^{2+} ion or haem derivatives in the cell may lead to chemical attacks on other cell components. A hypothesis that this is responsible for the action of isoniazid on *M. tuberculosis* is outlined elsewhere (Winder, 1959).

Many other readily oxidizable substances have chemical and biological effects which may be attributed to their auto-oxidation. For example, the metal-catalysed auto-oxidation of ascorbic acid oxidizes fatty acids (Geyer, Kydd & Ryan, 1957) and benzene (Ekman, 1944), destroys vitamin B_{12} (Rosenberg, 1956) and inactivates β-glucuronidase (Levvy & Marsh, 1957). Ascorbic acid is toxic to a wide range of organisms (Lwoff & Morel, 1942*a*, *b*; Strangeways, 1937), particularly when Cu^{2+} ion and hydrogen peroxide are added (Ericsson & Lundbeck, 1955), which would increase $HO^{\cdot}$ formation. The toxicity of certain sulphur compounds is probably due to their auto-oxidation (Woiwod, 1954; Taylor, 1957). These chemical and biological effects of auto-oxidation are presumably due to the formation of free radicals and other reaction intermediates.

Certain of the biological effects of metal ions such as Cu^{2+} and Fe^{3+} appears to be due to their ability to catalyse auto-oxidation. These two metals in particular increase the toxicity of a number of chelating agents for many micro-organisms (Sorkin, Roth & Erlenmeyer, 1952; Albert, Rees & Tomlinson, 1956; White, 1955). The chelating agents may facilitate auto-oxidation by these ions, or may themselves be auto-oxidized, or may facilitate penetration of the ions into the organisms where oxidation may be more destructive.

Characteristically, many auto-oxidizable substances are toxic under certain conditions and growth-promoters under others. If these substances can produce free radicals or other toxic auto-oxidation intermediates, and yet are capable of destroying free radicals produced otherwise (e.g. by ionizing radiation), this situation can be understood. However, interpretation of the results in any instance is difficult. Whether such a substance will function as a net producer or destroyer of free radicals will depend on many factors: the concentration of radicals produced otherwise, the concentration of the auto-oxidizable substance, the oxygen concentration, the metal ions present, the pH, the temperature, etc. This is also probably the explanation of why some of these substances may function either as mutagenic or anti-mutagenic agents.

SUMMARY

1. Mn^{2+} and Cu^{2+} ions were the most active catalysts found for isoniazid auto-oxidation at pH 7·5 and 37°. The velocity constant of the oxidation of 0·2 mM-isoniazid was 2·9 $hr.^{-1}$ when catalysed by 0·045 mM-Mn^{2+} in bicarbonate buffer, and 3·4 $hr.^{-1}$ by 0·045 mM-Cu^{2+} in borate buffer. Mn^{2+} ion was active down to 0·05 μM.

2. The activity of the catalysts depended on the buffer. Mn^{2+} ion was most active in phosphate or bicarbonate and Cu^{2+} ion in bicarbonate, borate or veronal.

3. Certain chelating agents increased the catalytic activity of Mn^{2+} ion when they were present at a lower molar concentration than it, but inhibited its activity at higher concentrations.

4. Hydrogen peroxide inhibited isoniazid auto-oxidation catalysed by Mn^{2+} ion, but stimulated that catalysed by Cu^{2+} ion.

5. Isoniazid auto-oxidation followed an approximately unimolecular course under certain conditions.

6. Hydrazides of a number of other acids also auto-oxidized.

7. *iso*Nicotinic acid, di*iso*nicotinylhydrazine and *iso*nicotinamide were products of auto-oxidation of isoniazid catalysed by either metal. Hydrogen peroxide resulted from auto-oxidation catalysed by Mn^{2+} ion. Free radicals, probably $OH^{\cdot}$ and radicals derived from isoniazid, were formed during auto-oxidation catalysed by Cu^{2+} ion.

8. Peroxidase did not affect isoniazid unless hydrogen peroxide and a phenol were present, when condensation products derived from isoniazid and the phenol were formed.

9. It is suggested that free-radical formation during auto-oxidation is a major factor in the toxicity of isoniazid and other auto-oxidizable substances.

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The Trisaccharide Fraction of Some Monocotyledons

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In those plants which accumulate fructosans (chiefly members of the Compositae and Campanulaceae, and the monocotyledons), oligosaccharide fractions of a similar character are always present. Relatively little is yet known about these fractions, despite the increased attention which has been given in recent years to the structure of the polysaccharides.

Dedonder (1952) and Bacon & Edelman (1951) suggested that the trisaccharide fraction of the tubers of *Helianthus tuberosus* contained *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(1→2) β-fructofuranoside (1^F-β-fructosylsucrose). Later work (Allen & Bacon, 1956) confirmed this, and also demonstrated the virtual absence of other trisaccharides. In unpublished work, the author has recently shown that the rampion (*Campanula rapunculus* L.) resembles the composite in this respect.

The use of paper chromatography has shown the presence of fructose-containing oligosaccharides in various monocotyledons: for example, in the leaves and stems of wheat (Bradfield & Flood, 1950; Lopatecki, Longair & Farstad, 1957), in onion bulbs (Egyptian 'Silvers'; Dr J. Edelman, personal communication, 1951), in leaves and stems of barley (Porter & Edelman, 1952) and in the seeds of cereals (MacLeod, 1951; White & Secor, 1953), but in very few cases have the substances been identified. Schlubach, Lübbers & Borowski (1955) have reported that *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(6→2) β-D-fructofuranoside (6^F-β-fructosylsucrose; 'kestose') is the main trisaccharide of perennial ryegrass (*Lolium perenne* L.).

It seemed that the use of chromatographic methods which have permitted the isolation of the products of fructose transfer by invertases (Edelman, 1956; Bacon, 1954) might help to characterize the trisaccharide fraction of monocotyledons. These trisaccharides are of interest not only because they serve as substrates for digestive enzymes, and for microbial attack above and below the ground, but also because their structures may act as pointers to the mechanism of fructosan biosynthesis (cf. Feingold, Avigad & Hestrin, 1956).

In the experiments reported here these methods were applied to the onion, *Allium cepa* L. (a preliminary report has appeared: Bacon, 1957), the leek, *A. porrum* L., and two grasses: Italian ryegrass (*Lolium multiflorum* Lam.) and tall oatgrass (*Arrhenatherum elatius* L., J. and C. Presl, var. *bulbosum*, 'onion couch').

Two trisaccharides, 1^F-β-fructosylsucrose and *O*-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→2) β-D-fructofuranoside (6^G-β-fructosylsucrose have been identified in all four species; 6^F-β-fructosylsucrose is probably present, but in smaller amounts, in both grasses.

MATERIALS

Onions and leeks. These were grown in 1956–57 in the gardens of this Institute, the varieties of onion being 'Crosslings' and 'Golden Mammoth', and of leek 'Scottish

Musselburgh'. Other samples of onions of unknown origin were purchased locally from time to time.

Grasses. Italian ryegrass, strain S 22, was grown in the greenhouse at about 18° in pots of garden soil.

Tall oatgrass was collected in hedgerows round Dittisham, South Devon, during July 1957. By this time the plants had lost their seeds and were almost air-dry. Only the basal nodes and lower part of the stem were kept.

Sugars. The trisaccharides used as reference standards were obtained from plant material (1^F-β-fructosylsucrose) or were the products of invertase action (6^G-β-fructosylsucrose and 6^F-β-fructosylsucrose). Dr B. H. Howard of the Rowett Institute (see Howard, 1959) kindly supplied us with a sample of the last-named.

METHODS

Extraction of plants. This followed closely that described by Bacon & Dickinson (1957). All material, with the exception of that from *A. elatius*, was extracted with boiling water as soon as possible after its removal from the plant, the pots of ryegrass being brought into the laboratory for sampling. After the addition of an equal volume of ethanol and removal of any precipitate by filtration, the extract was evaporated to dryness under reduced pressure and dissolved in water. At this stage, and during further fractionation, the soluble sugars were examined by paper chromatography.

Paper chromatography. Most of the solvents and spraying reagents used have already been described (Bacon & Dickinson, 1957); additional solvents are mentioned where their use is described below.

A useful reagent for free ketoses was brought to our notice by Dr D. J. D. Hockenhull of Glaxo Laboratories Ltd., Greenford, Middlesex; it was developed there for the detection of steroidal primary and secondary ketols (Brooks *et al.* 1958).

For use with sugars, 20 mg. of 2:5-diphenyl-3-*p*-styrylphenyltetrazolium chloride (May and Baker Ltd., Dagenham, Essex; M & B 1767) is dissolved in 10 ml. of ethanol and immediately before spraying is mixed with an equal volume of 0·1 N-NaOH. This reagent is more reactive than those based on 2:3:5-triphenyltetrazolium chloride (Trevelyan, Procter & Harrison, 1950; Wallenfels, 1950). After a few minutes at room temperature, or with slight warming, free ketoses and oligosaccharides having a ketose-reducing group appear as purple spots on a yellow background; 5 μg. of fructose per spot is detectable. Aldoses do not appear to react at all, unless the paper chromatogram is heated strongly during the removal of solvent. (This spraying reagent is referred to below as the 'TSTZ spray'.)

Column chromatography. The extracts were further fractionated on charcoal–Celite columns (cf. Bacon & Dickinson, 1957). Where the oligosaccharide component was small a preliminary separation on charcoal–Celite was carried out with water followed by 25% (v/v) ethanol, the ethanol eluate then being subjected to gradient elution from a similar column. Extract containing x mg. of total trisaccharide was eluted from columns of about $2x$ g. of charcoal–Celite (1:1, w/w) with a gradient produced by 50% (v/v) ethanol dropping into $20x$ ml. of water. This usually separated the trisaccharides into three fractions, but did not always separate them completely from accompanying di- and tetra-saccharides. For this reason each trisaccharide fraction was further purified by partition chromatography on Celite (Lemieux, Bishop & Pelletier, 1956). As much as 50 mg. of sugar was applied to 10 g. of Celite no. 535 packed in a column 19 mm. diam. and 10–11 cm. high, and eluted with butan-1-ol saturated with water. No special precautions were taken to control temperature but a trap was inserted between the main reservoir and the column to remove any droplets of water. The trisaccharides emerged after about 600–700 ml. had passed through the column and were contained in about 400 ml. of butanol. Evaporation to dryness under reduced pressure always left a greasy residue from which the sugar could be extracted with water. Attempts to remove the contaminating material by a preliminary extraction of the Celite with acetone, or with butanol in a Soxhlet apparatus, were unsuccessful, as also was a preliminary fractional distillation of the butanol.

Characterization of trisaccharides. Indications of the nature of the trisaccharides were given by their R_F values and response to various spraying reagents on paper chromatograms, and by their position in the effluent from charcoal columns (cf. Bacon, 1954). After separation the optical rotation was measured where the total amount available permitted, and analyses of fructose (Seliwanoff reaction; Bacon & Bell, 1948) and glucose (glucose oxidase; Huggett & Nixon, 1957) were made on oxalic acid hydrolysates.

The Raybin test (Raybin, 1933) was applied in solution or on paper (Breuer & Bacon, 1957). In the latter case it was found better to apply 0·5–1·0 mg. of sugar to paper previously soaked in 5% (w/v) $MgCl_2$ and dried, and to spray with a 1% (w/v) solution of diazouracil in 0·1 N-NaOH, freshly prepared at 0°. After 3 min. in an oven at 80° a positive reaction was discernible and the blue intensified when the papers were left for a day or two at room temperature.

The samples of 1^F-β-fructosylsucrose and raffinose isolated from Celite columns were crystallized without difficulty; 6^G-β-fructosylsucrose was converted into a dry powder by precipitation with ether from solution in methanol. The infrared spectra of these materials were examined and compared with those of authentic specimens.

Additional evidence for the presence of 6^G-β-fructosylsucrose was obtained by partial acid hydrolysis (Bacon, 1954), which produces 6^G-β-fructosylglucose, a reducing disaccharide with R_F between that of the trisaccharide and that of sucrose.

RESULTS

For each plant the isolation procedure followed the outline already given under Methods: from charcoal–Celite columns fractions which would be expected to contain respectively 6^F-, 1^F- and 6^G-β-fructosylsucrose were first obtained, and then, if necessary, were subjected to partition chromatography on Celite columns. A crystalline sample of 1^F-β-fructosylsucrose was obtained from each of the plants examined. In each case its infrared spectrum was found to be identical with that of an authentic sample, and the Raybin test was negative. Partial acid hydrolysis and the Raybin test

were applied to each sample suspected to be 6^G-β-fructosylsucrose, with the expected results. Infra-red spectra confirmed the identity for *A. cepa*, *A. porrum* and *L. multiflorum*; the sample from *A. elatius* was not examined. Additional quantitative information is given in Table 1, and features peculiar to each plant are considered below.

Onion (Allium cepa *L.*)

Paper chromatograms of extracts of the whole bulb showed the presence of considerable amounts of fructose-containing oligosaccharide. With the exception of a sample of Egyptian onions bought in 1957 the onions examined showed no sign of true fructose polysaccharide, all the oligosaccharide moving off the base line when development with butanol–acetic acid (Partridge, 1948) was continued for 4–5 days. Examination of a series of fractions from charcoal–Celite suggested that the largest molecules contained seven to eight monosaccharide residues.

Extraction of the bulb, layer by layer, showed that the oligosaccharide occurred mainly in the inner region; the outer scales often contained nothing but sucrose, fructose and glucose, and occasionally very little water-soluble carbohydrate at all. A typical analysis is given in Table 2. The total sugar and oligosaccharide concentrations varied markedly from top to bottom and round the circumference of each scale.

About 10% of the ketose of extracts was present in the trisaccharide fraction (i.e. about 0·5% on a fresh-weight basis). Raffinose was not seen at any stage of the fractionation. Only two trisaccharides could be detected, 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose (Table 1). The total yield corresponded to about 0·4% of fresh weight of onion taken, the 1^F-β-fructosylsucrose preponderating slightly.

The pattern of elution of the oligosaccharides from charcoal–Celite was complex, and there were at least two components of each chain length and possibly more. Only 1^F-β-fructosylsucrose emerged pure; 6^G-β-fructosylsucrose was accompanied by the first tetrasaccharide to emerge (fructose:glucose ratio, 2·95:1), and in later fractions more overlapping occurred. However, it appears that by a combination of absorption with partition chromatography it should be possible to separate each of the oligosaccharide fractions above trisaccharide into at least two fractions.

Table 1. *Some quantitative observations on the trisaccharide fractions*

Optical rotation was measured in a 2 dm. stainless-steel tube, capacity about 2·5 ml. Fructose and glucose were estimated on samples hydrolysed with 0·5% oxalic acid at 100° for 30 min.

Suspected structure	Species	Yields of fraction (mg./100 g. fresh wt. of tissue)*	$[\alpha]_D$ Calc. on dry wt.	$[\alpha]_D$ Calc. from sugars in hydrolysate	Ratio, fructose:glucose in acid hydrolysate
1^F-β-Fructosylsucrose	*A. cepa*	150, 235	+28°	+28°	1·90
	A. porrum	45, 75	+27	+28	1·94
	L. multiflorum	10	+23	+24	1·95
6^G-β-Fructosylsucrose	*A. cepa*	125, 165	—†	+21	1·80
	A. porrum	65	+19	+22	1·90

* To nearest 5 mg. Yield of 6^G-β-fructosylsucrose from *L. multiflorum*: 10 mg.; yield of each trisaccharide from dry stems of *A. elatius*: 15–20 mg.

† Not measured.

Table 2. *Composition of individual layers of onion bulb*

An onion (120 g.) was halved and the scales were separated. Each scale was weighed and extracted with twice its weight of water at 100°. The extracts were analysed by quantitative paper chromatography, the ketose being measured in regions corresponding to fructose, sucrose and as many of the higher oligosaccharides as could be separated. The results are expressed as mg. of ketose/g. fresh weight of tissue. The scales are numbered from the outside, 9 being the innermost part of the bulb, including the growing point.

Sugars	Scale no. 1	2	3	4	5	6	7	8	9
Pentasaccharide and higher	—	—	—	—	2·7	3·3	6·4	8·5*	12·0
Tetrasaccharide	—	—	—	—	4·7	4·6	4·0	8·7	10·1
Trisaccharide	—	—	—	0·5	6·2	7·4	7·4	9·5	4·7
Sucrose	4·7	4·6	5·2	5·6	10·4	11·4	9·6	11·1	12·3
Fructose	16·4	15·3	14·6	14·2	11·0	12·2	12·3	9·1	4·9
Wt. of scale (g.)	28·0	22·5	32·9	14·0	8·0	4·2	2·1	1·0	1·1

* Pentasaccharide 3·5; hexasaccharide and higher 5·0.

All the oligosaccharide was hydrolysed into glucose and fructose by yeast invertase, or by dilute oxalic acid at 100°.

Leek (Allium porrum *L.*)

Although onion and leek have basically the same structure, in the mature leek plant the leaves remain green and are less sharply demarcated from the bulbous portion. The water-soluble carbohydrates increase in concentration towards the base as the green decreases. When compared with the onion bulb, the leek bulb was found to contain similar concentrations of fructose, sucrose and lower oligosaccharide, but five to ten times as much higher oligo- and poly-saccharide. The proportion of the total ketose comprised in the higher polymers thus exceeds even that in the innermost layer of the onion analysed in Table 2, and was only approached (in our limited experience) by extracts of layers of the Egyptian onions mentioned above. The green portions of the leaves contained little oligo- or poly-saccharide, and raffinose could be seen to be a major component of the trisaccharide fraction. The distribution of oligo- and poly-saccharide through successive sheaths, judged qualitatively, resembled that in the onion bulb. Extracts of the bulbous portion were taken for an investigation of the trisaccharide fraction.

The polysaccharide did not interfere with fractionation on charcoal–Celite, but the first preparations of 1^F-β-fructosylsucrose would not crystallize. More careful examination of these effluents showed that the trisaccharide was immediately preceded by a substance of slightly greater R_F (butanol–acetic acid), containing only fructose and reducing the TSTZ spray. When these fractions were excluded, the 1^F-β-fructosylsucrose crystallized without difficulty. 6^G-β-Fructosylsucrose was isolated from later fractions of the effluent. A mixture of a tetrasaccharide and raffinose was present in the intervening fractions. The raffinose was isolated and crystallized, and its identity confirmed by examination of the infrared spectrum.

As in the onion, the oligosaccharide fraction was very complex, additionally so from the presence of raffinose and its higher homologues. Some evidence for the presence of the latter was obtained by subjecting extracts to mild acid hydrolysis and comparing the resistant fructose-free oligosaccharides with those present in a mild acid hydrolysate of psyllid honeydew from the ash tree, *Fraxinus excelsior* (Bacon & Dickinson, 1957). Stachyose was present (as judged by this incomplete evidence) in extracts from all parts of the plant, but verbascose could be detected, and there in very small amounts, only in the fleshy roots. The latter contained smaller amounts of sucrose and oligosaccharide than the leaves.

Italian ryegrass (Lolium multiflorum *Lam.*)

Examination of young plants (five to eight leaves) showed the presence of fructosan but very little oligosaccharide. Raffinose was conspicuous in the green part of the leaves but present only in traces in the sheaths. Since the latter have a higher content of water-soluble carbohydrate, extracts were made from plants from which all but the newest leaf had been severed at the junction of leaf with sheath. The material extracted was therefore essentially the sheaths of the older leaves and the whole of the youngest, perhaps 30–40 % of the weight of the plant (minus roots) and half of the total ketose.

After preliminary separation of the oligosaccharide fraction from mono- and poly-saccharide, quantities of extract corresponding to 35–45 g. fresh weight were applied to columns containing 20–40 g. of charcoal, and three portions of effluent were collected corresponding to the three fructosylsucroses. Corresponding fractions from several such fractionations, representing a total of 125 g. of grass, were combined for further examination.

The first fraction showed on paper chromatograms two main spots, resembling sucrose and 6^F-β-fructosylsucrose in R_F and colour reactions. The total amount of 6^F-β-fructosylsucrose in this fraction was very small, comparison with standards on paper chromatograms suggesting not more than 2 mg. The portion also contained smaller amounts of raffinose, and of two substances reducing the TSTZ spray, one having the same R_F as the substance found in leek extract.

The other two fractions (which both contained small amounts of raffinose) were chromatographed on Celite and yielded about 15 mg. each of 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose respectively. The specific rotation could not be measured accurately, but the other tests of identity were applied.

Tall oatgrass
(Arrhenatherum elatius *var.* bulbosum)

This species was examined because it accumulates fructosan as a reserve carbohydrate. The dry bulbous lower nodes of the stem (100 g.) were sliced into boiling water and the whole was later transferred to a blender to disintegrate the very tough residue. The extract so obtained contained large amounts of polysaccharide, part of which was not removed by precipitation with 50 % ethanol and subsequently contaminated the effluent from charcoal columns. Eventually the greater part was removed by raising the ethanol concentration to 80 % (v/v). The precipitate, corresponding to 63 g. of original stems, weighed 10·8 g. Part of it was

purified by precipitation with $Ba(OH)_2$ (Palmer, 1951), and the product, which had an ash content of less than 0·1 %, had $[\alpha]_D^{21} - 44°$. Analyses of fructose and glucose suggested that it was at least 97 % pure and contained 2·8 % of glucose. Colin & de Cugnac (1926) also found $[\alpha]_D - 44°$ for a fructosan from this species.

The oligosaccharide fraction was by comparison very small, but three fractions were obtained resembling closely those from ryegrass.

The first fraction, which was relatively more abundant than in the ryegrass, was equally complex. It contained an appreciable amount of sucrose. Paper chromatography in several solvents (butanol–acetic acid–water; butanol–pyridine–water, Jeanes, Wise & Dimler, 1951; propan-2-ol–ethanol–water, MacLeod & McCorquodale, 1958) and the application of various spraying reagents provided the following information about the trisaccharide component: (*a*) the main component contained glucose and fructose and had R_F values indistinguishable from those of 6^F-β-fructosylsucrose; (*b*) a substance reducing the TSTZ spray was also present; (*c*) hydrolysis with dilute oxalic acid left a disaccharide with R_F similar to that of melibiose.

Chromatography of the fraction on Celite confirmed that the main trisaccharide component corresponded with 6^F-β-fructosylglucose, but small amounts of substances with larger and smaller R_F values were also detected. The reducing substance was distinguishable from melibiose, from the reducing trifructose isolated from the leek extracts and from 6^F-β-fructosylsucrose by its behaviour in one or other ot the solvents named.

Only very small amounts of trisaccharide could be isolated from the other two fractions. From the second, a few milligrams of crystalline 1^F-β-fructosylsucrose were obtained and from the third a trisaccharide which was not fully characterized, but is probably 6^G-β-fructosylsucrose.

Observations on the metabolism of the onion oligosaccharides

By dividing a single scale of an onion bulb into many small pieces and selecting groups representative of all positions, it proved possible to obtain comparable samples. Unless these special precautions were taken it was impossible to distinguish metabolic changes from the great local variations in concentration of the carbohydrates already referred to above.

The vacuum-infiltration technique was used to introduce glucose and fructose, in 20 % (w/v) solution, into the bulb tissue. After infiltration the pieces were rinsed in water, blotted on filter paper and left in covered Petri dishes at room temperature for periods up to 3 days. During this time no colonies of micro-organisms were seen. (When dinitrophenol was added to the infiltration medium extensive growth of bacteria occurred in 1 day, suggesting that poisoning of the tissue facilitated their establishment on its surface.) The pieces were then extracted in boiling water and the ketose content of the oligosaccharides was measured by quantitative paper chromatography (Bacon & Loxley, 1952).

Although clearly the variability of the starting material still influenced the results, in the limited number of experiments done (12 with glucose and 2 with fructose) there was a substantial increase in sucrose and often a marked increase of the trisaccharide fraction; Table 3 gives the results of eight experiments of identical design, with glucose infiltration. In experiments in which higher oligosaccharides were also estimated [a total of five infiltrations with glucose, three with fructose and four with 20 % (w/v) sucrose] the ketose of this fraction also increased. Because the designs of these experiments varied it is not possible to combine the results, but the increases were of the order of 50 %, as with the trisaccharide fraction.

DISCUSSION

The oligosaccharides of the grasses constitute such a small proportion of the total carbohydrate that it might be argued that they arise as artifacts of the extraction process. The leaf material is so thin that it seems unlikely that any enzymes could survive long enough in the boiling water to bring about any appreciable transformation of the sucrose or polysaccharide, but the high temperature itself might cause some hydrolysis of the fructosan (Laidlaw & Reid, 1951; Schlubach, 1958). If this were so, one might expect to find mainly reducing

Table 3. *Changes in sugars of onion bulb after infiltration with glucose*

A single scale from an onion weighing 150–200 g. was divided into small pieces and three groups were selected (*A*, *B*, *C*), each of about 25–30 pieces (8–10 g.). *A* and *B* were infiltrated with 20 % (w/v) glucose and *C* with water. *A* was extracted at once with water at 100°, *B* and *C* after 3 days in a closed vessel (see text). Ketose was measured in the fructose, sucrose and trisaccharide fractions after separation on paper chromatograms. Results are expressed in mg. of ketose/g. fresh wt. of original tissue; the figures given are the averages of eight such experiments.

	Infiltrated with water; extracted after 3 days	Infiltrated with glucose	
		Extracted at once	Extracted after 3 days
Trisaccharide	4·3	3·7	5·6
Sucrose	4·7	5·8	10·5
Fructose	14·8	17·1	16·9

polyfructose fragments among the oligosaccharides. In fact very little reducing activity could be detected on paper chromatograms, despite the use of a sensitive reagent.

However, it is possible that the reducing substance (probably a trifructose) contaminating the 1^F-β-fructosylsucrose fractions from the leek arose from some hydrolysis of polysaccharide, but not necessarily at the initial extraction stage. Extracts of leek tissue made by blending in the presence of mercuric chloride solution at room temperature contained less material reacting with the TSTZ reagent than those made in water at 100°. However, the main reducing substance in boiling-water extracts, although apparently liberated by hydrolytic conditions (e.g. during the removal of butanol–acetic acid from paper chromatograms), does not seem to be a carbohydrate; it has a greater R_F than the trisaccharides and gives little reaction with the benzidine–trichloroacetic acid spray (Bacon & Edelman, 1951). Most of the leek extracts prepared showed little reducing activity associated with the main oligosaccharide series; the one from which the trifructose was isolated was exceptional in this respect, and may have suffered some hydrolysis during concentration.

In the onion, where no polysaccharide fraction is found, there can be no doubt that the oligosaccharides are present as such in the intact bulbs, and it is tempting to argue that the similarity in the trisaccharide fractions of all four species shows that they occur as a result of normal physiological processes. If this is accepted we should expect 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose to be the typical trisaccharides of the monocotyledons, whereas 6^F-β-fructosylsucrose would occur only occasionally or in relatively small amounts. This picture is confirmed by the paper-chromatographic studies of White & Secor (1953) on the oligosaccharide fraction of the wheat berry (6^F-β-fructosylsucrose was present in very small amounts, insufficient for analysis), but it is not easily reconciled with the results of Schlubach *et al.* (1955). They found only 6^F-β-fructosylsucrose ('kestose') in the trisaccharide fraction of *Lolium perenne* L. Their description of their fractionations does not permit one to speculate on the possible presence of other trisaccharides, except in one respect: they state that their trisaccharide gave a positive Raybin reaction. A positive reaction was wrongly attributed to 6^F-β-fructosylsucrose by Albon, Bell, Blanchard, Gross & Rundell (1953) (see Bacon & Bell, 1953; Howard, 1959), so that 6^G-β-fructosylsucrose, which does react in this test, may have been present in their material. The preponderance of 6^F-β-fructosylsucrose apparently shown by their work might be explained by a species difference, or a considerable change in the trisaccharide fraction as the plants mature. We have not attempted to study the physiological influences on the composition of the trisaccharide fraction. The complexity of the fraction makes this difficult, but by no means impossible; estimation by quantitative paper chromatography of the ketose-containing trisaccharide in each of the three fractions from charcoal–Celite would give a good idea of how the amounts of the three main trisaccharides vary during growth and maturity. Such a study might resolve the contradiction between our results and those of Schlubach and his collaborators.

Although in the composites there is only one trisaccharide, and this, 1^F-β-fructosylsucrose, has the 2:1-fructose–fructose link characteristic of the polysaccharide fraction (inulin, etc.), in the monocotyledons we have examined the situation is more complex. In the first place there are two or three fructosylsucroses, and secondly that one which has the 2:6-linkage characteristic of grass and cereal fructosans is by no means the most prominent, and is seemingly absent in the leek and onion. A parallel to this situation is found in the oligosaccharide fraction present in levansucrase reaction mixtures (Feingold *et al.* 1956; Avigad & Feingold, 1957). Here 1^F-β-fructosylsucrose is the most abundant trisaccharide and 6^F-β-fructosylsucrose is barely detectable, although the 2:6-linkage is also the predominant linkage of the bacterial levan.

This parallel could be taken to indicate that the grass fructosans are synthesized by an enzyme of the levansucrase type. Two other facts must, however, be taken into account in any discussion of synthetic mechanisms. The first is that 6^G-β-fructosylsucrose is also present in appreciable amounts, recalling the transfer products of leaf invertases (Allen & Bacon, 1956). Henderson, Morton & Rawlinson (1959) have recently shown that in all probability the invertase in the pulp of the banana (*Musa cavendishii* Lamb.) is responsible for the appearance of trisaccharide during the ripening process. They identify the trisaccharide as 6^G-β-fructosylsucrose, but all their criteria would apply equally well to a mixture of this substance with 1^F-β-fructosylsucrose (for example, we have found that the Raybin test is positive on such mixtures), which would be a more likely product of a higher plant invertase (Allen & Bacon, 1956). Such action of invertase *in vivo* could explain the trisaccharide fraction of monocotyledons, but not the oligosaccharide fraction as a whole, because the leaf invertases which have been examined produce mainly trisaccharides, only traces of tetrasaccharide and nothing of higher molecular weight. There is also the consideration that in the onion both the trisaccharide and the higher oligosaccharide fractions increase in amount when the sucrose

content is raised by infiltration of glucose or fructose.

A stronger argument for differences between levansucrase action and fructosan biosynthesis in monocotyledons is the absence of polysaccharide from most of the onions we examined. This might suggest that there are two synthetic processes, one producing oligosaccharide and the other polysaccharide, which sometimes occur together, sometimes separately. A closer study of the synthesis of fructose oligosaccharides in the onion might therefore not provide direct evidence for the mechanism of fructosan synthesis, as we suggested earlier (Bacon, 1957), but could nevertheless help towards a better understanding of fructose metabolism in this very important group of plants.

The work of Hestrin and his collaborators (Hestrin, Feingold & Avigad, 1956) has shown that the low-molecular-weight fructosides discussed here are relatively poor acceptors for transfructosylation by levansucrase. It is therefore necessary to be cautious in suggesting that 6^G-β-fructosylsucrose can serve as a basis for fructosan synthesis, but if it did it would give rise to non-terminal glucose residues and might explain the isolation of trimethylglucose from hydrolysates of methylated grass levans (cf. Harwood, Laidlaw & Telfer, 1954).

An extension of the same argument would connect the absence of 6^G-β-fructosylglucose from *Helianthus tuberosus* with the apparent absence of non-terminal glucose residues from inulin (Aspinall & Telfer, 1953). Further caution is needed here though, because of the relatively limited range of inulin-type fructosans that has so far been examined in detail. Thus Chollet (1947) has isolated from various *Campanula* species a polysaccharide very similar to inulin but having $[\alpha]_D$ as low as $-29°$ (cf. $[\alpha]_D - 40°$ or even higher for inulin). We have isolated a similar polysaccharide from *Campanula rapunculus* having $[\alpha]_D - 27°$ and a glucose content of 5·2%. Whether such wide differences in specific rotation can be explained by modifications of a structure in which glucose occupies a terminal position must be a matter for further investigation.

The position of raffinose and related oligosaccharides in the monocotyledons examined is of some interest. We could find no evidence for its presence in extracts of any part of the onion plant, whereas it was present throughout the leek tissues. If, as has been suggested, it plays the part of a reserve carbohydrate in some plants (Pridham, 1958) and of a sugar of translocation in others (Zimmermann, 1958), it is possible that in the onion the fructosan-type oligosaccharide and sucrose are themselves able to fulfil these functions.

SUMMARY

1. The oligosaccharide fractions from *Allium cepa*, *A. porrum*, *Lolium multiflorum* and *Arrhenatherum elatius* have been fractionated by adsorption chromatography on charcoal–Celite and partition chromatography on Celite columns.

2. Two trisaccharides, 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose, have been identified in all four extracts. The two grasses also contained smaller amounts of a trisaccharide which was tentatively identified as 6^F-β-fructosylsucrose.

3. These findings are discussed in relation to possible mechanisms of fructosan biosynthesis in plants.

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Protein Interactions and Metabolic Response to Stimulating Agents in Isolated Cerebral Tissues: Histones as Inhibitors

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After keeping slices of cerebral tissues in buffered glucose media at 0° for 5–17 hr., their normal increase in respiration in response to electrical pulses is largely lost; on examining a variety of naturally occurring materials for ability to restore the response, fractions of blood plasma were found most active (Marks & McIlwain, 1959). The restoring agent appeared to be protein in nature and fraction IV-4 of Cohn *et al.* (1946), which contained α- and β-globulins, was most active.

The present investigation began with the supposition that fraction IV-4 might interact at a chemical level with a constituent of cerebral tissues. Though the possible types of interaction are very wide, it was decided first to see whether fraction IV-4 gave an obvious change, as of light absorption or precipitation, with material extracted from cerebral tissues. A material which forms a precipitate with fraction IV-4 has been found in cerebral extracts; it appears to be a histone. Moreover, the cerebral extracts which give the precipitates, and also basic proteins from other sources, have been found to be inhibitors of the response of cerebral tissues to stimulating agents.

These findings enable mechanisms to be suggested for the loss of excitability on keeping the tissue at 0°, and for its restoration, and these mechanisms are explored further below.

EXPERIMENTAL

Tissue metabolism. The procedures described by Marks & McIlwain (1959) were followed in preparing tissues from the cerebral cortex of guinea pigs, in applying electrical pulses and high concentrations of potassium salts, and in measuring respiration and lactic acid formation. Tissues were incubated at 37·5° in an oxygenated medium containing (mM): NaCl, 134; KCl, 5·4; $CaCl_2$, 2·6; $MgSO_4$, 1·3; KH_2PO_4, 1·2; glucose, 10 and glycylglycine 30, and adjusted to pH 7·4 with NaOH. This is termed the glycylglycine medium; additions to it are noted in individual experiments below.

Extracts of cerebral tissues. Centrifuging was carried out at 0° with either an MSE machine or Spinco model L ultracentrifuge. Extracts (*a*) and (*b*) were made with cold medium as described by Marks & McIlwain (1959) but buffered with glycylglycine, and extract (*c*) was the 'hot extract' of Marks & McIlwain (1959), made with water.

For extracts (*d*) to (*i*), guinea pigs were killed, the whole brain (3 g.) was removed quickly and put into an ice-cooled homogenizer tube, weighed, 6 ml. of 0·9% NaCl was added and the mixture was ground vigorously for 30 sec. Coarse structures were removed by running the mixture through glass wool, and 15 min. later the filtrate was centrifuged for 10 min. at 10 000 *g*, giving a supernatant (*d*). The precipitate was suspended in 12 ml. of water at 0° and after 30 min. centrifuged as before, giving a supernatant (*e*) and a residue which was resuspended as before; centrifuging for 30 min. then gave not a clear solution but a persistent sol (*f*) as supernatant; the remaining precipitate (*g*) was suspended in glycylglycine medium for test.

After preparing extract (*d*) from other guinea pigs, the residue (from 6·6 g. of brain) was suspended in 20 ml. of 0·2N-H_2SO_4 and left at 0° for 60 min., centrifuged for 10 min. at 2000 *g* and the residue was extracted as before. The combined supernatants were tied in Visking cellulose tubing and rotated mechanically in 2 l. of water, renewed at intervals of 1½ hr. for 4½ hr., yielding (*h*), the non-dialysable material. This, already neutral, was evaporated at 10 mm. Hg to a few millilitres for test. Extract (*i*) was prepared in the same way as extract (*h*), but commencing with the residue from extract (*d*) from 7 g. of brain, which by the addition of 5M-NaCl and water was made to 14 ml., 2·6M in

NaCl; 28 ml. of ethanol was added, and after 60 min. the mixture was filtered and the filtrate dialysed and evaporated as described before.

For extracts (*j*) to (*m*), guinea-pig brain was ground as for (*d*), but in 0·25 M-sucrose containing 0·5 mM-ethylenediaminetetra-acetic acid (EDTA), and fractions were prepared by differential centrifuging according to the methods of Heald (1959) based on those of Brody & Bain (1952) and McMurray, Berry & Rossiter (1957). Extract (*j*) was derived from material sedimenting between 1500 and 104 000 *g*, and extract (*k*) from material sedimenting at 1500 *g*, in each case according to the method used in making extract (*i*) described above. The NaCl yielded an extremely viscous, glairy or mucus-like sludge with the material sedimenting at 1500 *g*, subsequently with ethanol giving a clear solution and a precipitate in sticky shreds, easily filtered through a fluted paper. For the other extracts, the 'crude nuclear' fraction used in making extract (*k*) was further separated by centrifuging above M-sucrose. This fraction, prepared from 5 g. of guinea-pig brain and suspended in 10 ml. of 0·25 M-sucrose with 0·5 mM-EDTA, was carefully run above 20 ml. of M-sucrose in tubes for the Spinco head no. 25, with minimal disturbance of the solution. After 1 hr. at 90 000 *g* this yielded a lighter fraction, between the two layers of sucrose, containing most of the free nuclei and a heavier layer below the M-sucrose. The two portions were placed in separate tubes and suspended in 4 ml. of 0·2 N-H_2SO_4, and after 30 min. at 0° the tubes were centrifuged (5000 *g*) for 10 min. The supernatants were kept and extraction of the residues was repeated until the extracts no longer formed a precipitate or opalescence with a saturated aqueous solution of picric acid. This required three repetitions with the lighter material, yielding (combined) extract (*l*), and one with the heavier fraction, yielding extract (*m*). The extracts were dialysed and evaporated as was extract (*h*). During the dialysis some insoluble material separated, as in comparable preparations of Smillie, Marks & Butler (1955), but tests with picric acid showed the bulk of the material precipitating with this reagent to remain in the solution after dialysis.

Other materials. We are indebted to Mr D. Purdie of Armour Laboratories, Eastbourne, for supplies of ox-blood plasma proteins prepared by ethanol precipitation at controlled pH, according to Cohn *et al.* (1946). Globin was kindly given by Wellcome Laboratories. Histone from calf-thymus nuclei was obtained from L. Light and Co. Ltd. (specimen I) and Nutritional Biochemicals Corp. (specimen II), and protamines (salmine and clupein) were from L. Light and Co. Ltd. (specimen I of salmine) and from British Drug Houses Ltd. (specimen II).

Properties of the extracts. Dry weight was determined after heating samples for 2 hr. at 100°, absence of further change being confirmed after a further 1 hr. at 100°. For observation of the reaction with blood-plasma fraction IV-4, this was shaken with glycylglycine medium (50 mg./ml.) for 1 hr., centrifuged at 2000 *g* and the supernatant used. The tissue extract (0·1 ml.), with or without an equal volume of medium, was placed in a 2 ml. tube with a pointed end, and the IV-4 solution added down the side of the tube in 10 μl. portions until up to 50 μl. had been added, an interval of 3–4 min. being allowed between additions. Precipitates which were formed were often at interfaces, and in some cases dissolved in excess of IV-4; visual impressions of their maximal extent were recorded.

Reaction with picric acid was sought by adding to 0·1 ml. of extract, in tubes as described above, 0·1 ml. of a saturated aqueous solution of picric acid. In some cases (indicated) an additional 0·1 ml. of the extract was taken and N-acetic acid added until no more precipitate formed; the tube was then centrifuged and the picric acid solution added.

RESULTS

Properties of cerebral extracts

The cerebral extracts first prepared were (*a*)–(*h*) of Table 1. When examined at concentrations of a few mg./ml., two extracts gave a slight opalescence with the blood-plasma fraction IV-4; these extracts had been made by allowing the material to stand in glycylglycine medium, and by boiling in water. Only extract (*h*), made by extracting with H_2SO_4 material which was insoluble in iso-osmotic NaCl, gave an appreciable precipitate with fraction IV-4. All eight extracts were then examined for action on the respiration of cerebral tissues, both in a normal, unstimulated condition and also when stimulated by applied electrical pulses. Extracts made in cold medium, in hot water or by grinding in iso-osmotic NaCl markedly accelerated the respiration of the tissue under normal conditions; respiration with pulses was also in some cases accelerated. The extract made with 0·2 N-H_2SO_4 was again unusual in lowering the respiration of the electrically stimulated tissue; it did not have its greatest effect immediately, but after pulses had been applied for 20–30 min.

The method of preparing extract (*h*) indicated that the materials reacting with fraction IV-4 and inhibiting the tissue's response were not easily dialysable and were not extracted from the tissue by iso-osmotic salts or by water, but were extracted by acid. Basic lipids or proteins appeared likely to be present; an alternative method which extracted basic proteins from other tissues was therefore applied, 2·5–3·0 M-NaCl and ethanol being used (see Mirsky, 1943; Butler, Davidson, James & Shooter, 1954; Crampton, Lipshitz & Chargaff, 1954; Crampton, Stein & Moore, 1957; Smillie *et al.* 1955). This yielded extract (*i*), which again formed a precipitate with fraction IV-4 and caused delayed inhibition of the respiration of electrically stimulated tissues (Table 1).

The association of basic proteins with cell nuclei being known, cerebral tissues were next ground and differentially centrifuged before extraction (Table 2). By application of the NaCl–ethanol extraction to the different particulate fractions it was evident that the nuclear fractions differed from others in their swelling with 2·5–3·0 M-NaCl. They yielded material reacting with

fraction IV-4, and again inhibited the electrically stimulated respiration of cerebral tissues, the inhibition increasing with time. The histone-like material could also be extracted from nuclear fractions by 0·2 N-H_2SO_4. When samples of the extracts (*a*)–(*m*) were acidified with acetic acid, centrifuged and picric acid was added to the supernatant solutions (see Experimental section), heavy precipitates were given with extracts (*h*), (*i*), (*k*), (*l*) and (*m*), which reacted with fraction IV-4, and also with (*a*) and (*b*); lesser precipitates were obtained with (*d*), (*e*) and (*j*) and none with (*c*) and (*f*).

Table 1. *Properties of cerebral extracts*

Reaction with fraction IV-4 was carried out as described in the Experimental section. In examining action on tissue respiration, the extracts were present initially in 3·5 ml. of glycylglycine medium, the tissue was added and respiration measured during 40 min. at 37° without applied pulses. Condenser pulses of peak potential 10 v and time constant 0·4 msec. were then applied at 100/sec. and respiration was measured for a further 40 to 60 min. Values are the means of duplicate or triplicate determinations agreeing to within 6%, except where standard deviation and (in parentheses) number of values are quoted.

Extract (see Experimental section)	Prepared from tissue	Reaction with fraction IV-4 of ox-plasma proteins	Effect on respiration: Dry wt. of extract added (mg.)	Fresh wt. of brain from which extract was derived (mg.)	Respiratory rate (μmoles of O_2/g./hr.): Before pulses	With pulses
None	—	—	—	—	62±2 (6)	120±5 (6)
(*a*)	Cut; leached cold	Slight opalescence	5*†	250	83	112
(*b*)	Cut; leached cold	None	9·8*†	250	96	122
(*c*)	Cut; boiled	Slight opalescence	6·5*	250	80	138
(*d*)	Ground; supernatant	None	8·6*	400	73	121
(*e*)	Ground; water extract	None	8·8	400	58	125
(*f*)	Ground; soluble	None	12·3	500	64	128
(*g*)	Ground; insoluble	None	22·2	500	63	119
(*h*)	Ground; H_2SO_4	Precipitate	19·0	500	59	96, 80‡
			5·7	150	60	106, 84‡
(*i*)	Ground; NaCl–ethanol	Precipitate	18	920	68	118, 86‡
			7·2	370	65	128, 92‡
			1·8	92	65	120, 110‡

* These extracts were examined also at concentrations one-third and one-fifth of those quoted, but were without action on respiration.

† Corrected for weight of constituents of medium used in extraction.

‡ Rates during the second 20–30 min. of application of pulses; when a second rate is not noted in this column, respiration did not change.

Table 2. *Properties of extracts from particulate fractions obtained by centrifuging ground guinea-pig brain*

Extracts were prepared as described in the Experimental section, and tested and recorded as described in Table 1.

Expt.	Fraction	Extract and method of preparation	Reaction with fraction IV-4	Effect on respiration: Dry wt. of extract added (mg./ml. of medium)	Fresh wt. of brain from which extract was derived (mg./ml. of medium)	Respiratory rate (μmoles of O_2/g./hr.): Before pulses	With pulses: First 20–30 min.	With pulses: Second 20–30 min.
1	Fine particles	(*j*) NaCl–ethanol	None	2·9	215	64	121	116
				1·0	86	61	118	116
1	Nuclear fraction	(*k*) NaCl–ethanol	Precipitate	3·1	215	63	113	83
				1·2	86	62	122	104
2	Light nuclear fraction	(*l*) H_2SO_4	Precipitate	2·2	500	56	102	62
				0·7	167	59	100	70
2	Heavier nuclear fraction	(*m*) H_2SO_4	Precipitate less than from (*l*)	1·5	500	59	100	82
				0·5	167	60	104	92
1 and 2	None	—	—	—	—	62±2 (4)	120±4 (4)	113±4 (4)

Basic proteins as inhibitors

The relationships just described were explored by examining the effects of a number of other protein preparations on the metabolism of isolated cerebral tissues (Table 3). Histones from calf-thymus nuclei readily reproduced the inhibition of respiratory response found in cerebral preparations. They were active at concentrations as low as 0·1 mg./ml., and their effect again increased during the application of pulses (Table 3). Much greater concentrations (2·5 mg./ml.) were without action on the tissue's normal respiration; the action on the stimulated tissue extended also to glycolysis, for the additional lactic acid which accumulated when pulses were applied was less in the presence of the histone.

Protamine preparations proved even more active than histones. Clupein and different salmine preparations showed the same selective inhibition of electrically stimulated respiration and glycolysis at concentrations between 10 and 500 μg./ml. Moreover, a protamine preparation also inhibited respiration and glycolysis when these were stimulated by high concentrations of potassium salts, a situation showing many analogies to electrical

Table 3. *Histone and protamine preparations as inhibitors of metabolic changes induced by electrical pulses*

The substances examined were added to glycylglycine medium before the tissue was placed in it. Respiration was measured, electrical pulses were applied and results recorded, as described in Table 1. At the end of the experiment samples of medium were taken for determination of lactic acid. Histone and protamine preparations: see Experimental section.

Substances added and concn. (mg./ml.)		Respiratory rate (μmoles of O_2/g./hr.) Before pulses	With pulses	Lactic acid (μmoles/g./expt.)
None		57	(No pulses)	41±4 (4)
None		58±2 (8)	117±3 (8)	90±5 (4)
Histone I	0·5	61	78, 56*	67
	0·33	60	85, 69*	—
	0·1	58	86, 72*	71
Histone II	0·33	60	70, 55*	63
	0·15	62	107, 90*	—
	0·03	62	120, 110*	—
Salmine I	0·5	56	35	51
	0·1	59±1 (5)	67±4 (5)	48
	0·03	57	84	60
	0·01	56	103	69
Salmine II	0·5	56	54	43
	0·1	57	54	57
Clupein	0·5	58	39	—
	0·1	58	53, 48*	—
	0·05	58	95	—
	0·01	58	114	—
None		62	(No pulses)	42
None		63	112†	56
Clupein	0·1	62	73†	37
Fibrinogen	0·5	58	126	—
	0·1	60	113	—
Gliadin	1·0	58	119	90
	0·5	58	136	—
	0·2	63	131	84
	0·1	58	119	—
Glutenin	1·0‡	60	112	79
	0·2	60	117	84
Globin	1·5‡	62	110	70
	0·3	61	120	99
Plasma albumin	3·0	60	118	83
	0·6	62	119	—

* This value refers to the final 20–30 min. of the experiment and is given when the rate at that time differs from the rate (quoted first) when pulses were first applied.

† No pulses were applied but KCl giving 50 mM-K^+ ions was added from a side arm.

‡ The glutenin specimen was dissolved at an alkaline pH and globin at an acid pH, but at these concentrations partial precipitation took place in the neutral experimental media.

stimulation of the cerebral tissues (McIlwain, 1952; Wollenberger, 1955). Many other proteins were without action on respiration and glycolysis of isolated cerebral tissues, with or without stimulating agents, and when examined in concentrations up to 100 times those in which the protamines acted (Table 3).

Time relationships. The protamine and histone preparations were effective as inhibitors only after they had been in contact with cerebral tissues for some time. In the experiments of Table 3, tissues were incubated with the proteins for a total time of 50 min. (during 40 min. of which respiration was measured) before pulses were applied. When pulses were applied after only 15 min. incubation of tissue with a protamine preparation, the action of the protamine was at first smaller but increased with continued incubation in the same fluid (Table 4). The initial incubation period of 50 min. appeared sufficient to exhibit the maximal action of protamine; it was, however, insufficient for the full action of calf-thymus histone, the effect of which increased between 50 and 120 min. while pulses were applied. This phenomenon was shown also by the inhibitory material, probably a histone, prepared from the nuclear fraction of cerebral tissues.

In other experiments of Table 4, tissues were exposed to protamine-containing media for a limited time only. After 15 min., incubation media (some with and some without protamine) were replaced by fresh media, in all cases lacking protamine. When no protamine had been present, replacement led to respiratory rates some 10% below those normal to the tissues. When a protamine-containing medium was replaced by one lacking protamine, marked inhibition of response was found but the inhibition was smaller than that found when incubation continued in protamine-containing media. With pulses applied at 50 min., inhibition remained at about the levels previously seen when pulses were applied at 15 min. Apparently penetration of protamine into the tissue or combination with the tissue took place progressively during the first 50 min. of incubation.

Interactions with plasma-protein fraction IV-4. All the protamine and histone preparations of Table 3 readily yielded precipitates with fraction IV-4 in neutral solutions in water and in the medium employed in tissue metabolism. Precipitation occurred with a range of concentrations of IV-4, which included the 2–10 mg./ml. at which the fraction is effective in restoring respiratory response to cerebral tissues which have lost response through keeping at 0° (see above). When fresh cerebral tissues were examined in a medium to which had been added an inhibitory concentration of protamine together with fraction IV-4 at 10 mg./ml., the tissue's response was at its normal, high, uninhibited value (Table 5). The combination of IV-4 and protamine was presumably sufficiently firm to render ineffective at least 90% of the protamine. Moreover, when protamine in potentially inhibitory concentrations was present together with cerebral tissues for the first 15 min. of such experiments, it was still possible to annul almost completely its effect by subsequent addition of fraction IV-4. Similarly, after tissues had been incubated in histone-containing media for 15 min.,

Table 4. *Time relationships in the action of a protamine preparation*

Experiments were carried out and results expressed as described in Table 1, except as specified below. When medium was replaced, vessels were taken from their manometers; medium was removed by a Pasteur pipette, the vessel and tissue (still in its grid electrode) were rinsed with 1 ml. of glycylglycine medium and this also was removed; 3·5 ml. fresh medium was added and the vessels were re-equilibrated with oxygen. These operations took 15–20 min. with a group of six vessels.

Clupein present initially (mg./ml.)	Course of experiment (min.)	Respiratory rate (μmoles of O_2/g./hr.)		
		Before pulses	First 30 min. with pulses	Second 30 min. with pulses
0	Pulses applied at 15 or 50 min.	59±3 (6)	118±5 (6)	114±3 (6)
0·1	Pulses applied at 15 min.	—	91	61
0·25	Pulses applied at 15 min.	—	62	40
0·1	Pulses applied at 50 min.	58	58	48
0·25	Pulses applied at 50 min.	57	56	48
0	Medium replaced after 15 min. by medium without clupein; pulses applied at 50 min.	55±3 (4)	102±6 (4)	—
0·1	Medium replaced after 15 min. by medium without clupein; pulses applied at 50 min.	52	79	—
0·25	Medium replaced after 15 min. by medium without clupein; pulses applied at 50 min.	58	66	—

the addition of fraction IV-4 allowed a much greater respiratory response (Table 5).

In view of the finding of the previous section that protamines and histones are delayed in coming into action, the experiments just described did not indicate with certainty whether fraction IV-4 was capable of removing the effects of an established association between inhibitor and tissue. To investigate this, the last experiment of Table 5 was carried out. After the 15 min. incubation of tissues with protamine-containing media, these media were replaced with media lacking protamine, but some of which contained fraction IV-4. The tissue with the IV-4 regained most of its ability to respond to pulses.

DISCUSSION

Basic proteins as inhibitors. The histone and protamine preparations are impressively potent as inhibitors of the response of cerebral tissues to stimulating agents. The preparations used are undoubtedly mixtures of different molecular species, several fractions having been obtained from calf-thymus histone and molecular weights between 10 000 and 30 000 suggested for them (Stedman & Stedman, 1951; Phillips, 1958; Luck, Rasmussen, Satake & Tsvetikov, 1958; Mauritzen & Stedman, 1959). Protamines have also been fractionated and molecular weights of about 5000–8000 proposed (Craig, Konigsberg, Stracher & King, 1958; Ross, 1954). Thus the lowest concentrations at which the excited cerebral tissues were markedly affected, of 0·03 mg. of salmine/ml. or 0·1 mg. of calf-thumus histone/ml., correspond to about 2–10 μM. In the present system barbiturates have effects of comparable magnitude at 300 μM, cocaine at 20 μM and chlorpromazine, among the most potent inhibitors, at 5 μM (McIlwain & Greengard, 1957; Bollard & McIlwain, 1959).

Evidence connecting the effects of added histones with the relatively unresponsive state induced in cerebral tissues by standing in cold media is as follows. (1) Of a variety of cerebral extracts, the few which diminished the tissue's response to stimulation were made by methods known to yield histones, and gave reactions of histones. Inhibitory material was obtained from nuclear preparations of cerebral tissues. Nuclear preparations from cerebral tissues have been found to contain histones similar to those obtained from other somatic cells of the body (Mirsky, 1943; Palladin, Rashba & Shtutman, 1951). (2) The blood-plasma protein fraction IV-4, found to restore responsiveness to cerebral tissues after keeping in cold media (Marks & McIlwain, 1959), restored responsiveness also to fresh tissues whose response had been inhibited by protamines and histones. Fraction IV-4 formed precipitates with the protamines and histones, and with the inhibitory cerebral extracts, at the pH and in the media in which its actions as a restoring agent are exhibited. The formation of protein–protamine complexes is markedly dependent on pH (Ross, 1954), exhibiting relationships with the isoelectric points of the components, and the properties of fraction IV-4 as a restoring agent may in part depend on such characteristics. (3) The unresponsive condition was previously found to be induced simply by keeping the tissue cold in a chemically defined medium normally adequate for response. Responsiveness was not restored by addition of material appearing in the medium, nor was loss of

Table 5. *Antagonism of blood-plasma fraction IV-4 to the action of protamine and histone preparations*

Additions at 15 min. were made by tipping from side arms. Replacement of media was carried out as described in Table 4 and after replacement tissues were incubated for 40 min. without applied pulses, and for 40 min. with pulses; other details were according to Table 1.

Additions to medium before incubation (mg./ml.)	Change at 15 min. incubation	Respiratory rate with pulses (μmoles of O_2/g./hr.)
None	None	118
None	Addition of IV-4 (5 mg./ml.)	121
Histone I (0·33)	None	85, 69*
Histone I (0·33)	Addition of IV-4 (5 mg./ml.)	111, 99*
Salmine I (0·1)	None	67±4 (5)
Salmine I (0·1) and IV-4 (10)	None	127
Salmine I (0·1)	Addition of IV-4 (10 mg./ml.)	101
Salmine I (0·1)	Addition of IV-4 (2 mg./ml.)	98
Salmine I (0·1)	Addition of IV-4 (0·4 mg./ml.)	68
None	Media replaced by fresh portion of medium without salmine	110
Salmine I (0·15)		60
Salmine I (0·15)	Medium replaced by medium without salmine and with IV-4 (10 mg./ml.)	94

* Rates during the second 20 min. of application of pulses. When a second rate is not quoted in this column, respiration did not change.

responsiveness affected by adding a variety of adsorbents to the cold media with the tissue. This suggests the change to be within the tissue rather than by loss to the medium, or exchange with the medium.

Possible migration of tissue histone. If the tissue's histone is indeed involved in producing the unresponsive state induced by being kept cold, it would appear necessary for the histone to leave its normal site in the cell nuclei, where it is pictured to be in combination with deoxyribonucleic acid. This appears a relatively loose combination, as emphasized by Mirsky (1943) and illustrated by the methods of extraction used above. A salt-like combination is proposed, though with some specificity between particular histone and nucleic acid components (Crampton, 1957); this is, however, based on the properties of the extracted components. In certain abnormal conditions, histological changes have been noted in cerebral tissues which would be consistent with migration of the type now postulated. Nuclei have been observed to stain with increased intensity with basic dyes, and material staining with acid dyes to appear elsewhere in the cell (Cajal, 1909; Wertham & Wertham, 1934). In the present case, the histone must be pictured as reaching a site involved in the tissue's reaction to electrical pulses, and in view of the basic nature of the histone an acidic site appears likely as a target. Major possibilities are thus the anionic sites or carriers postulated in ion-transport mechanisms or the phosphoproteins involved in the phosphate transfers which take place during the tissue's response to electrical pulses (Heald, 1959). Protamines and histones have also been reported as both activators and inhibitors of enzyme reactions (Krebs, 1954; Madsen & Cori, 1954; Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954).

It must in addition be supposed that the protamines and histones reach such sites when added to media containing normal cerebral tissues. Protamines and histones have been concluded to enter cells of a variety of types (Fischer & Wagner, 1954), and time relationships now observed in the action of the protamines and histones presumably indicate slow penetration of the tissue or its cells. It was noted previously (Marks & McIlwain, 1959) that for cerebral tissues to become unresponsive on keeping at 0° for a few hours some addition was required of the fluid medium normally used in incubation. A relatively small volume, no more than that of the tissue itself, sufficed. Cerebral tissues in such fluids *in vitro* swell under adverse conditions. Absorption of up to two-thirds of their volume of fluid can occur, the swelling being partly intracellular and partly extracellular (Pappius & Elliott, 1956). Possibly the additional fluid or some resulting disorganization gives additional opportunity for migration of substances such as the histone; more direct evidence for this is being sought. Instances of migration of proteins from tissue slices to surrounding fluids, or from one intracellular site to another, have been reported (Stern, Eggleston, Hems & Krebs, 1949; Adams & Burgess, 1959).

SUMMARY

1. Extracts made from cerebral tissues by a variety of methods were examined for action on respiration of guinea-pig cerebral cortex incubated in glucose-containing media. An extract made with acid was found to contain non-dialysable material which inhibited the tissue's response to applied electrical pulses, though having little effect on respiration in the absence of pulses.

2. It was concluded that the inhibitory material is probably a histone. Similar material was obtained by sodium chloride–ethanol extraction, and by extraction of nuclear fractions of the tissue. Histone and protamine preparations from other sources were also inhibitory, being active when incorporated in media at about 5 μM. They inhibited also the tissue's glycolytic response to pulses, as well as its glycolytic and respiratory responses to 50 mM-potassium salts.

3. The blood-plasma fraction IV-4 which restored response to cerebral tissues after they had been rendered unresponsive by being kept at 0° restored response also to tissues treated with protamine and histone. Fraction IV-4 formed precipitates with the basic proteins, and also with the inhibitory cerebral extracts, but not with a number of non-inhibitory cerebral extracts.

4. Time factors in the action of the protamine and histone preparations were investigated. It is suggested that the tissue's histone is involved in the loss of excitability which occurs when cerebral tissues are kept in cold media.

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The Isolation of Lombricine and its Possible Biological Precursor

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In connexion with experiments on the chemical and biological synthesis of 2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate (lombricine) it was desirable to have, as a reference compound, adequate amounts of the naturally occurring material. The isolation of lombricine from the earthworm (*Lumbricus terrestris*) has been described by Van Thoai & Robin (1954), but the low yield reported stimulated a search for an improved method. The simplified procedure described in this paper results in much greater yields of analytically pure material. In addition, experiments are described on the detection and characterization of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate which, it is suggested, is the immediate precursor of lombricine.

MATERIALS AND METHODS

All reagents used were analytical-grade and solvents used for chromatography were purified by fractional distillation. The reference compounds, 2-guanidinoethyl phosphate, 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate (serine ethanolamine phosphodiester, SEP) and lombricine, were synthesized by D. I. Magrath & I. M. Beatty (unpublished work), who kindly provided samples for chromatographic purposes. Those containing serine were synthesized from the L-isomer. All other compounds were commercial preparations. The reference standards used were chromatographically homogeneous in all solvent systems employed.

The earthworms used were collected in the Canberra area and were of mixed species but were predominantly *Allolobophora caliginosa* and *Octolasium cyaneum*.

The method described by Rosenberg, Ennor & Morrison (1956) for the estimation of arginine was used to determine the concentration of guanidino compounds present in earthworm extracts. These compounds will be referred to as 'guanidine-reacting material'.

Chromatography. All exploratory chromatograms were run in ascending fashion on washed no. 3 Whatman paper, 28 cm. × 28 cm., unless specifically mentioned. Washing was carried out by immersing the ends of the papers in a tank of water and allowing the water to run off for several hours after the front had reached the bottom edges. Two-dimensional chromatograms involving reinforcement with reference compounds were run on pairs of papers cut from one sheet, and care was taken to develop each in the same sequence of solvent systems and in the same direction relative to the original sheet. This was found to produce identical patterns in any one pair of solvent systems.

When chromatography was employed for preparative purposes, solutions of the unknown compounds were applied to the origin as 3–5 mm. wide bands on sheets 60 cm. × 60 cm. The requisite band was located with the aid of markers, cut out and eluted by the method of Reith (1957). This enabled elution from a band approx. 200 cm.^{2} in area in a volume of about 1 ml.

The solvent systems employed, together with the approximate development time, at 23° for ascending chromatograms on paper 28 cm. × 28 cm. were:

I. Water-saturated phenol	13–14 hr.
II. Butan-1-ol–acetic acid–water (5:3:2, by vol.)	8–9 hr.
III. Acetone–acetic acid–water (2:2:1, by vol.)	3–4 hr.
IV. Di*iso*propyl ether–acetic acid–ethanol–water (4:4:1:2, by vol.)	8–9 hr.
V. Methyl Cellosolve (2-methoxyethanol)–water (9:1, by vol.)	8–9 hr.
VI. Methanol–formic acid–water (8:1:1, by vol.)	4 hr.
VII. Ethanol–formic acid–water (7:1:2, by vol.)	8–9 hr.
VIII. Ethanol–M-ammonium acetate buffer, pH 3·8 (7:3, v/v)	7–8 hr.
IX. Methyl Cellosolve–butan-2-one–acetic acid–water (15:40:6:24, by vol.)	8–9 hr.
X. *tert.*-Butanol–butan-2-one–water–diethylamine (10:5:10:1, by vol.)	18 hr.
XI. Water-saturated phenol–ethanol–aq. ammonia (sp.gr. 0·88)–water (150:40:10:1, by vol.)	12 hr.

Guanidine derivatives and inorganic phosphate were detected on chromatograms as described by Rosenberg (1959*a* and 1959*b* respectively).

Amino acids and related compounds were detected by dipping the paper in a 0·2% solution of ninhydrin in acetone, followed by heating in the usual manner.

RESULTS

Isolation of lombricine

Live earthworms were frozen in liquid air and stored at −60°. The frozen material (1 kg.) was disintegrated in 200 g. batches in a Waring Blendor with 300 ml. of 0·75 N-$HClO_4$. The suspensions were filtered by the aid of suction and the filter pads from each batch re-extracted in the Blendor with 200 ml. of extractant. The combined filtrates amounted to 3 l. Tests carried out on a third extraction revealed the presence of only small amounts of guanidine-reacting material and the residues were discarded. The combined extracts were brought to, and maintained at, 75° for 15 min. to hydrolyse any *N*-phosphorylated guanidine which may have been present. The extract, which contained considerable amounts of inorganic matter and glycogen in colloidal solution, was then cooled to 20° and treated with an equal volume of ethanol. Immediate precipitation occurred and clarification was achieved by passage through a Sharples centrifuge. The residue which contained no guanidine-reacting material was discarded and the clear, straw-coloured supernatant was passed at a rate of 14 ml./min. through a column (70 cm. × 4 cm.) of Zeo-Karb 225 (H^+ form) which retained all the guanidine-reacting material. The effluent was discarded and the column washed (20 ml./min.) with 1 l. of 0·01 N-HCl in 50% ethanol. The washings, which were yellow in colour but which contained no trace of guanidine-reacting material, were discarded.

The material retained on the column was eluted with aq. 2·5 N-NH_3 (flow-rate 11 ml./min.). The entire guanidine-reacting material was collected in 650 ml. and the major proportion of the NH_3 removed by distillation at 25° under reduced pressure. The solution (pH 10) was then diluted with water to 2 l. and passed through (5 ml./min.) a column (50 cm. × 312 cm.) of Dowex-2 (OH^- form). The effluent, which contained arginine, but no lombricine, as determined by paper chromatography in solvent system VII, was discarded. The column was then washed with water (1 l.) and the lombricine eluted with N-HCl (5 ml./min.) in a total volume of 700 ml. An equal volume of ethanol was added and this solution again passed at 10 ml./min. through a column (40 cm. × 4 cm.) of Zeo-Karb 225 (H^+ form). The entire amount of guanidine-reacting material was retained and the effluent was discarded. The column was washed with 0·01 N-HCl in 50% (v/v) ethanol and eluted with aq. N-NH_3 in a total volume of 500 ml. The eluate was distilled under reduced pressure at 25° to remove NH_3 and its volume reduced to 250 ml. The resultant solution was adjusted to pH 6·5 by the addition of 5 N-HCl and a small amount of suspended material removed by centrifuging. The solution was freeze-dried, the bulk of the solid redissolved in water in a final volume of 25 ml. and the small amount of insoluble material again removed by centrifuging. Ethanol (1 vol.) was added and the resultant cloudy suspension clarified by filtration. Additional ethanol (3 vol.) was then added and crystallization commenced immediately. The mixture was cooled to 3° and stored at this temperature for 3 hr. before filtration. The filtrate was retained and examined for the presence of SEP (see below). The crystals were redissolved in about 15 ml. of water and recrystallized by the cautious addition of ethanol (3 vol.). The crystals (white needles) were recovered by filtration, washed with ethanol and acetone and dried *in vacuo* over P_2O_5. Yield 650 mg., m.p. 224° (decomp.). [Found (for lombricine): C, 26·5; H, 5·71; N, 20·64; P, 11·6. $C_6H_{15}O_5N_4P$ requires C, 26·6; H, 5·5; N, 20·8; P, 11·5%.]

The product was checked chromatographically in solvent systems I and VII. It was free from other guanidines but contained barely detectable traces of ninhydrin-positive material which ran ahead of lombricine.

Detection of the possible lombricine precursor

The proposed structure of lombricine (Van Thoai & Robin, 1954) suggests that the corresponding amine, SEP, may be the biological precursor of lombricine, which could be formed from the former compound by a transamidination reaction involving arginine. Such a suggestion has already been made (Ennor & Morrison, 1958). Because of the similar nature of the acidic and basic groups of the two compounds, SEP was expected to behave in a manner similar to that of lombricine in the isolation procedure and to be present in the mother liquor from which the initial yield of lombricine was obtained. This liquor was therefore examined for the presence of SEP.

The mother liquor was treated with acetone (4 vol.) and allowed to stand overnight at 3°. The cloudy suspension was centrifuged and as the supernatant contained comparatively little ninhydrin-positive material it was discarded. The gummy precipitate was dissolved in 50 ml. of water, and the solution brought to boiling point, treated with charcoal and filtered. The filtrate was freeze-dried, the residue dissolved in 2 ml. of water and a small amount of flocculent precipitate removed by centrifuging. A sample of this solution was analysed by two-dimensional chromatography in solvent systems V and I. On treatment with ninhydrin, at least 16 spots were detected and one (R_F 0 in solvent V and 0·34 in solvent I) behaved similarly to a synthetic sample of SEP. This spot was also reinforced when SEP was added before development.

Samples of the solution were then chromatographed in a number of solvents, and it was found that in solvent system VII the major proportion of the ninhydrin-positive material ran with R_F 0·5. Since in this system authentic SEP had R_F 0·2, it was used to achieve a partial purification of the material contained in the mother liquor by preparative chromatography (see Methods).

A sample of the partially purified material was then checked chromatographically (solvent system I). Only three ninhydrin-positive spots were detected. The fastest-running component (R_F 0·55), which also gave a positive 1-naphthol–butane-2:3-dione reaction, was identified as lombricine. The intermediate component (R_F 0·34) corresponded in position to SEP, but the third component (R_F 0·22) was not identified. In view of this separation the remainder of the material was purified by chromatography in solvent system I and obtained in a final volume of 0·4 ml. This solution was then examined chromatographically in a number of solvent systems, and in all showed the presence of only one ninhydrin-reacting component with an R_F identical with that of an authentic sample of SEP (Table 1).

A sample of the solution was subjected to two-dimensional chromatography in solvent systems I and V. Similar chromatograms were developed with samples of authentic SEP and a mixture of the latter and the isolated material. In each experiment only one ninhydrin-reacting spot was obtained and its position was precisely similar in all chromatograms. The intensity of the colour reaction with the mixture was considerably greater than with either sample alone.

Hydrolysis. Samples of the eluate (0·04 ml.) were heated in sealed tubes at 105° for 30 min., 2 hr. and 4 hr. with 0·06 ml. of 10N-HCl. The hydrolysates were evaporated to dryness on watch-glasses heated on a steam bath. The residues were exposed to NH_3 to neutralize traces of residual HCl and dissolved in water (0·05 ml.). Samples of these hydrolysates were spotted on papers, together with a sample of authentic SEP which had been similarly treated and markers of serine, 2-aminoethyl serine phosphate, 2-aminoethyl phosphate and orthophosphate. The chromatogram was developed in solvent system II and then treated with ninhydrin. The ninhydrin-reacting areas were outlined in pencil and the chromatogram was treated with the appropriate reagents to render visible the inorganic phosphate and phosphorus-containing compounds. The chromatogram (Fig. 1) indicates that, in addition to inorganic phosphate, three ninhydrin-reacting compounds were released by acid hydrolysis. Two of these compounds corre-

Table 1. *R_F values of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and related compounds*

Solvent system (see Methods)	R_F						
	Serine	Serine phosphate	Ethanolamine	2-Aminoethyl phosphate	Lombricine	SEP	Isolated compound
I	0·43	0·12	0·53–0·69	0·46	0·55	0·34	0·34
II	0·44	0·23	0·53	0·29	0·23	0·15	0·15
III	0·62	0·44	0·84	0·55	0·31	0·22	0·22
IV	0·49	0·31	0·62	0·41	0·26	0·23	0·22
V	0·17	0·04	0·44	0·06	0	0	0
VI	0·57	0·42	0·68	0·40	0·18	0·13	0·12
VII	0·64	0·42	0·75	0·46	0·28	0·20	0·19
VIII	0·46	0·22	0·70	0·44	0·23	0·19	0·20

sponded in R_F to the serine and ethanolamine markers, but the fastest-running compound was unidentified. A similar pattern was revealed when a chromatogram was developed in solvent system X (Fig. 2).

Although the identity of the isolated material seems to be established, it was nevertheless thought desirable to gain more information about the fastest-running ninhydrin-reacting compound. Consequently samples of the serine, serine ethanolamine phosphodiester and 2-aminoethyl phosphate markers were heated with 6N-HCl under conditions similar to those used for the hydrolysis of SEP. These solutions were evaporated to dryness and taken up in a small volume of water as before. Samples of these solutions were applied to papers, together with the appropriate markers and hydrolysates of SEP and the isolated material. The chromatograms were developed in solvent systems II, VIII, X, XI and treated with ninhydrin and phosphate reagents. The results with the first

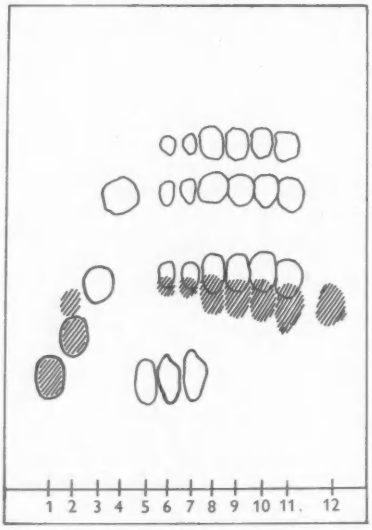

Fig. 1. Products of acid hydrolyses of authentic 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and isolated material. Outlined areas, ninhydrin-reacting material; shaded areas, phosphorus-containing material. Under the conditions employed SEP was not hydrolysed and thus the phosphorus moiety was not demonstrable. 1, Serine phosphate; 2, 2-aminoethyl phosphate; 3, serine; 4, ethanolamine; 5, SEP; 6, 8 and 10, 30 min., 2 hr. and 4 hr. hydrolysates of authentic SEP respectively; 7, 9 and 11, 30 min., 2 hr. and 4 hr. hydrolysates of isolated material; 12, inorganic phosphate. Solvent system II.

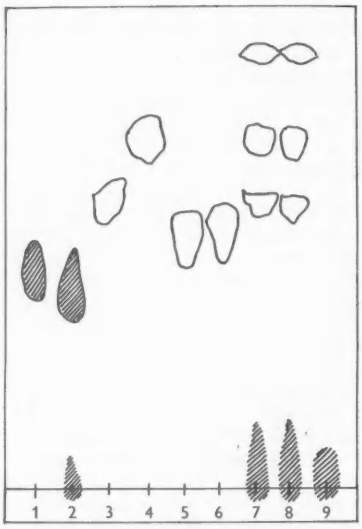

Fig. 2. Products of acid hydrolyses of authentic 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and isolated material. Outlined and shaded areas as in Fig. 1. 1, Serine phosphate; 2, 2-aminoethyl phosphate; 3, serine; 4, ethanolamine; 5 and 6, authentic SEP and isolated material respectively; 7 and 8, as for 5 and 6 but after 4 hr. acid hydrolysis; 9, inorganic phosphate. Solvent system X.

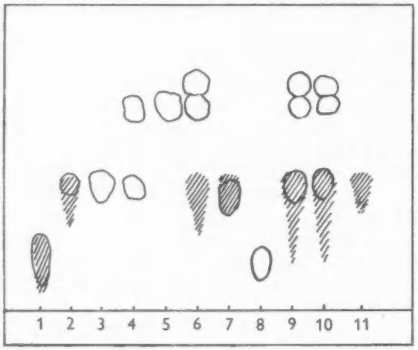

Fig. 3. Products of acid hydrolyses of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and related compounds. Outlined and shaded areas as in Fig. 1. 1, Serine phosphate; 2, serine phosphate hydrolysed; 3, serine; 4, mixture of serine and ethanolamine, acid-treated (see text); 5, ethanolamine; 6, 2-aminoethyl phosphate hydrolysed; 7, 2-aminoethyl phosphate; 8, SEP; 9 and 10, hydrolysates of authentic SEP and isolated material respectively; 11, inorganic phosphate. Solvent system VIII.

three solvent systems were similar; that for solvent system VIII is reproduced in Fig. 3. Serine and ethanolamine were unchanged after acid treatment and serine phosphate yielded the two expected products, serine and inorganic phosphate. However, with 2-aminoethyl phosphate the products were inorganic phosphate, ethanolamine and another ninhydrin-reacting compound which ran ahead of ethanolamine and corresponded in position to the fastest-moving component arising from the hydrolysis of SEP and the isolated material.

When developed in solvent system XI the chromatogram (Fig. 4) confirmed the above results, but in this system the fast-running component, produced as a result of the hydrolysis of 2-aminoethyl phosphate, SEP and the isolated material, was not detected.

On theoretical grounds it seems likely that chloroethylamine could be formed according to the following reactions:

$$H_2N \cdot CH_2 \cdot CH_2 \cdot O \cdot PO_3H_2 \rightarrow H_3PO_4 + H_2N \cdot CH{:}CH_2$$
$$H_2N \cdot CH{:}CH_2 + HCl \rightarrow H_2N \cdot CH_2 \cdot CH_2Cl$$

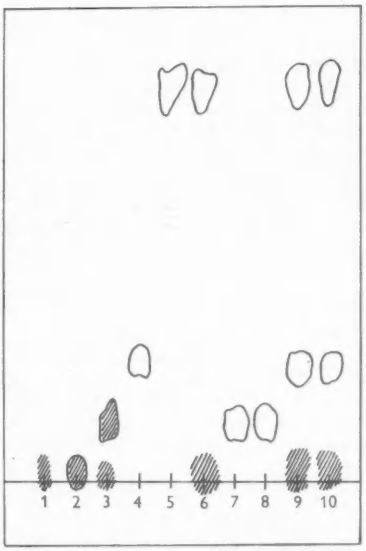

Fig. 4. Acid hydrolysates of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and 2-aminoethyl phosphate. Outlined and shaded areas as in Fig. 1. 1, inorganic phosphate; 2, serine phosphate; 3, 2-aminoethyl phosphate; 4, serine; 5, ethanolamine; 6, 2-aminoethyl phosphate hydrolysed; 7 and 8, authentic SEP and isolated material respectively; 9 and 10, as for 7 and 8 respectively, but after 4 hr. acid hydrolysis. Solvent system XI.

A sample of chloroethylamine was prepared and its chromatographic behaviour was identical with that of the fast-running component when examined in three solvent systems.

Guanylation. A sample (0·04 ml.) of a solution containing about 1 mg. of SEP/ml. was mixed with 0·02 ml. of a solution of *O*-methyl*iso*urea (2 mg./ml.). Sufficient 2 N-NaOH was added to render the mixture just alkaline to thymolphthalein and the mixture was allowed to react for 24 hr. at room temperature. A sample (10 μl.) of the neutralized reaction mixture was chromatographed in solvent systems I and IX and then sprayed with the 1-naphthol–butanedione reagent or treated with ninhydrin. Apart from *O*-methyl*iso*urea two 1-naphthol–butanedione-positive spots were detected. One of these was present in just detectable amounts and was not identified, but the other, which constituted the bulk of the guanidine-reacting material, was identified as lombricine by comparison with the natural material. Only two ninhydrin-positive spots were detected: one corresponded to SEP and the other to lombricine.

DISCUSSION

The present identification of SEP in extracts of earthworm tissue depends on several lines of evidence: the similarity in behaviour of the material with SEP when subjected to two-dimensional chromatography and when unidimensionally chromatographed in a number of solvent systems, the chromatographic similarity of the products of acid hydrolysis with those of the synthetic material, and finally the fact that guanylation of the isolated compound yielded a product similar in behaviour to natural lombricine. Beatty & Magrath (1959) have established that lombricine may be synthesized in high yield by guanylation of synthetic SEP with *O*-methyl*iso*urea. Theoretically guanylation of SEP could yield two products in the absence of protective groups on the α-NH_2 but, as Beatty & Magrath (1959) found, one product is formed almost exclusively. It would thus seem that the α-NH_2 group is unreactive under the conditions employed.

The appearance of serine and ethanolamine as a result of hydrolysis of SEP was to be expected and confirms the findings of Roberts & Lowe (1954). These latter findings were, however, not confirmed in that, in the present experiments, (*a*) it did not prove possible to detect the phosphate esters of ethanolamine or serine and (*b*) a third ninhydrin-reacting spot with an R_F greater than that of ethanolamine was detected. From the preliminary results obtained it seems probable that this spot is β-chloroethylamine.

The present experiments give no indication whether SEP isolated from earthworms contains

$$H_2N\cdot C(:NH)\cdot NH\cdot[CH_2]_3\cdot CH(NH_2)\cdot CO_2H + H_2N\cdot[CH_2]_2\cdot O\cdot PO(OH)\cdot O\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$$

Arginine — 2-Aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate

$$\downarrow$$

$$H_2N\cdot C(:CH)\cdot NH\cdot[CH_2]_2\cdot O\cdot PO(OH)\cdot O\cdot CH_2\cdot CH(NH_2)\cdot CO_2H + H_2N\cdot[CH_2]_3\cdot CH(NH_2)\cdot CO_2H$$

Lombricine — Ornithine

D-serine, as with lombricine (Beatty, Magrath & Ennor, 1959). If, however, SEP is the precursor of lombricine, it would be expected to contain D-serine, contrasting sharply with the SEP isolated from turtle muscle which has been reported to contain the L-enantiomorph (Roberts & Lowe, 1954). Experiments now in progress should determine this point.

The detection of SEP in extracts of worm tissue is only the second occasion on which this compound has been found in Nature. It was first isolated by Roberts & Lowe (1954) from extracts of turtle muscle, where it is believed to exceed 50 mg./100 g. Roberts & Lowe (1954) showed conclusively that L-serine was present in the molecule and the suggested structural formula was confirmed by synthesis (Jones & Lipkin, 1956).

SEP has not yet been shown to be the biological precursor of lombricine, and it is possible that the amidine group is introduced at an earlier stage of the biosynthesis. This, however, seems unlikely and several considerations suggest that SEP is the precursor: it is present in extremely small amounts, as might be expected of a biological intermediate; and it gives rise, by non-enzymic guanylation, to a product indistinguishable from natural lombricine.

The amidine group of the latter may arise from arginine by transamidination, as shown by the reaction at the top of this page.

Arginine appears to be the biological source of this group in all the naturally occurring guanidines so far investigated (cf. Ratner & Rochovansky, 1956). Experiments are now in progress to detect, in earthworm tissue, a transamidinase capable of transferring the amidine group from arginine to SEP to yield lombricine. The biological routes by which both lombricine and SEP are synthesized are also under investigation.

SUMMARY

1. A simple method for isolating 2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate (lombricine) from natural sources has been described. The method results in good yields of analytically-pure crystalline material.

2. 2-Aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate has been detected in earthworm extracts and it is suggested that this compound is the biological precursor of lombricine. The precursor was identified by the preparation of a guanylated derivative, by degradative procedures and by comparison with the synthetic product.

Our thanks are due to Dr D. I. Magrath for a sample of β-chloroethylamine, to Dr Joyce Fildes, Department of Medical Chemistry, for the microanalyses and to Mr B. Thorpe for skilled technical assistance.

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Steps in the Reactions of Chymotrypsin with Tyrosine Derivatives

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Chymotrypsin catalyses the hydrolysis of peptide, amide and ester bonds of many *N*-acylated amino acids, but it exhibits special specificity for compounds of aromatic amino acids (for a summary of chymotrypsin substrates, see Foster & Niemann, 1955). The esters and amides of other aromatic acids, such as methyl hydrocinnamate (Snoke & Neurath, 1949), are also hydrolysed by this enzyme and its specificity for compounds containing a ring structure is further emphasized by its ability to

bind and hydrolyse aliphatic esters of phenols such as *p*-nitrophenyl acetate (Hartley & Kilby, 1954).

We have suggested that the reactions of chymotrypsin with its substrates involve three steps (Hammond & Gutfreund, 1955; Gutfreund & Sturtevant, 1956*a*, *b*). The first of these steps is a rapid adsorption of the substrate on to the specificity site of the enzyme, the second involves a chemical reaction resulting in the acylation of the catalytic site of the enzyme and the third step involves the hydrolysis of the acyl-enzyme (see Scheme 1).

$$E+AB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{Compound I}}{E\text{–}AB} \xrightarrow{k_2} \underset{\text{Compound II}}{EA+B} \xrightarrow{k_3} E+A+B$$

Scheme 1

Both an hydroxyl group of serine and an imidazole group of histidine are essential components of the catalytic site of chymotrypsin; the diverse evidence for this has been summarized by Jandorf & Michel (1957). Hartley & Kilby (1954) and Gutfreund & Sturtevant (1956*a*, *b*) have shown that, at least in reactions with *p*-nitrophenyl acetate, the acylated enzyme is a chemical intermediate with a real existence and that this compound is formed by an imidazole-catalysed acylation of a serine hydroxyl group of the enzyme. The spectroscopic evidence of Dixon & Neurath (1957), which indicated that an imidazole group of the enzyme was acylated during the reaction, was shown to be misleading when Spencer & Sturtevant (1959) demonstrated that the acetylated enzyme studied by Dixon & Neurath (1957) was not on the pathway of the enzyme reaction.

The evidence for the existence of an acyl-enzyme intermediate and for the acylation of a serine hydroxyl group depended on the results of studies with *p*-nitrophenyl acetate, a rather special substrate which is only hydrolysed slowly by chymotrypsin. It has been suggested by Bernhard & Gutfreund (1958) that different enzyme–substrate intermediates might occur during the reaction of chymotrypsin with substrates which are more like its natural ones, namely compounds of aromatic amino acids. The present study with the nitrophenyl ester of *N*-benzyloxycarbonyl-L-tyrosine was suggested to us when Martin, Golubow & Axelrod (1959) prepared this compound and used it for the assay of chymotrypsin. The purpose of the experiments reported here was to find whether there is any evidence for the existence of an *N*-benzyloxycarbonyl-tyrosyl–enzyme intermediate (compound II of Scheme 1) during the reaction of chymotrypsin with *N*-carbobenzoxy-L-tyrosine nitrophenylate, and, if such a compound exists, to find which group or groups of the enzyme are involved in its formation. It will be shown that an *N*-benzyloxycarbonyl-tyrosyl–enzyme intermediate is formed, but its formation is so rapid that its kinetics cannot be analysed and the chemistry of the formation of compound II with this substrate has therefore not been elucidated.

EXPERIMENTAL

Buffers. Potassium phosphate solutions (0·05M) containing 20% (v/v) of propan-2-ol, pH 7·2 (as measured with a glass electrode), were used as solvent in all reaction mixtures.

Enzyme. The chymotrypsin stock solution was prepared by activation of crystalline chymotrypsinogen (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) by the method of Northrop, Kunitz & Herriott (1948). Recrystallized enzyme was stored at 2° in a solution of pH 5·0. It was assayed by measuring the initial rapid liberation of nitrophenol on addition of a portion of the enzyme to a solution (1 mM) of nitrophenyl acetate (Hartley & Kilby, 1954). The data of Biggs (1954) were used for calculation of the concentration of nitrophenol from the extinction at 400 mμ and pH. The stock solution was adjusted to be 2 mM with respect to active enzyme.

Substrates. *p*-Nitrophenyl acetate was kindly supplied by Dr W. N. Aldridge; the *p*-nitrophenyl ester of *N*-benzyloxycarbonyl-L-tyrosine (CTN) was kindly supplied by Dr C. J. Martin (Martin *et al.* 1959).

Spectrophotometric techniques. The results reported in this paper relied on the photometric observation of the rate of appearance of *p*-nitrophenol during the hydrolysis of *p*-nitrophenyl esters. Observations on a time scale of minutes were carried out in a Unicam spectrophotometer (SP. 500) at 400 mμ. The rapid-reaction measurements, on a time scale of fractions of a second, were carried out in a stopped-flow machine (Gibson, 1954) as modified by Gutfreund (1955) for the observation of the rate of appearance of *p*-nitrophenoxide ions during enzyme reactions. The magnitude of the deflexions of the oscilloscope beam were calibrated against standard solutions of known *p*-nitrophenol concentration. It was found that over the concentration range of *p*-nitrophenol used (0–50 μM) the instrument gave a linear response to concentration changes, which were calibrated in cm. deflexion per 10 μM-*p*-nitrophenol. Since all the solutions used were of the same pH and in the same solvent the proportion of *p*-nitrophenol in the ionized, coloured form was constant.

The time base of the 'solarscope' oscilloscope (Solartron Electronic Group Ltd., Thames Ditton, Surrey) was used for the determination of reaction velocities. Photographs of the oscilloscope tracings were projected in an enlarger and drawn on graph paper together with markers which gave a direct measure of the enlargement factor from oscilloscope screen to graph paper.

RESULTS

The Michaelis parameters for the reaction of chymotrypsin with CTN have been determined by Martin *et al.* (1959), who obtained a maximum overall rate at pH 8·0 and 30° of 480 sec.$^{-1}$ and K_m 32 μM. From four measurements of the initial

velocity we obtained the range of 100 ± 10 sec.$^{-1}$ for the steady-state turnover of the enzyme at 25°, pH 7·2 and CTN concentration 40 μM. Correcting for the suboptimum conditions of pH and substrate concentration from the graphs given by Martin *et al.* (1959) we obtain a maximum turnover of 300 sec.$^{-1}$ at 25° which is in reasonable agreement with the value of 480 sec.$^{-1}$ at 30°. Fig. 1*A* gives the record of one experiment carried out with a reaction mixture of final enzyme and substrate concentrations 0·4 μM and 40 μM respectively; this gives an example of the time course of the chymotrypsin-catalysed hydrolysis at relatively low enzyme concentration, suitable for calculations of the turnover number. Fig. 1*B* gives the record of an experiment at high enzyme concentration (20 μM); the substrate concentration and the concentration scale is the same as that in Fig. 1*A*, but the time scales of the figures are different. Comparison of the two records in Figs. 1*A* and *B* indicates that in the experiment at high enzyme concentration approximately half the total *p*-nitrophenol has been liberated by the time observation commenced. This is reminiscent of the observations of Hartley & Kilby (1954), who found on a very much slower time scale that during the relatively slow reaction between chymotrypsin and *p*-nitrophenyl acetate there occurred an almost instantaneous liberation of 1 mole of *p*-nitrophenol/mole of chymotrypsin. Fig. 2 gives a plot of the concentration of *p*-nitrophenol observed immediately after mixing, for a series of reaction mixtures with different concentrations of chymotrypsin. As can be seen, an essentially instantaneous liberation of a molar equivalent of *p*-nitrophenol occurs on the addition of chymotrypsin to CTN solutions. A model experiment was carried out to obtain some evidence about the time taken between mixing and the start of the record of the extinction of the reaction mixture in the stopped-flow device used for the above-mentioned experiments. The reduction of flavine mononucleotide by sodium dithionite was chosen for this purpose for two reasons. Identical optical conditions to those suitable for the observation of *p*-nitrophenol can be used, and since the reaction is second-order its half-time can be varied by variation of the dithionite concentration. As the concentration of dithionite was at least 100 times that of flavine mononucleotide, pseudo first-order

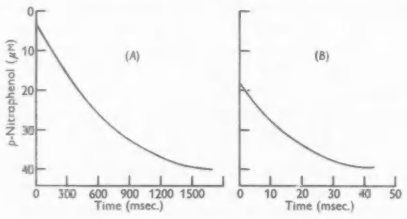

Fig. 1. Records of two observations of the time course of the liberation of *p*-nitrophenol during the reaction between chymotrypsin and the *p*-nitrophenyl ester of *N*-benzyloxycarbonyl-L-tyrosine (40 μM) at 25° in 20% (v/v) propan-2-ol, 0·05 M-potassium phosphate, pH 7·2. Chymotrypsin concentrations: (*A*) 20 μM; (*B*) 0·4 μM. All concentrations given are those in the final reaction mixture. Note different time scales for (*A*) and (*B*).

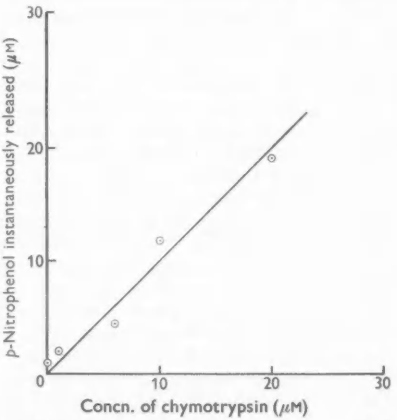

Fig. 2. Plot of the chymotrypsin concentration against the quantities of *p*-nitrophenol liberated before observation (3 msec.) during reactions between chymotrypsin and the *p*-nitrophenyl ester of *N*-benzyloxycarbonyl-L-tyrosine (40 μM) at 25° in 20% (v/v) propan-2-ol, 0·05 M-potassium phosphate, pH 7·2. All concentrations given are those in the final reaction mixture.

Table 1. *Kinetic constants for the chymotrypsin-catalysed hydrolysis of three tyrosine derivatives*

The observed Michaelis constant for the overall reaction $(K_m)_2$ is given as M and the rate constants in sec.$^{-1}$. The overall rate constant is k_0; k_2 and k_3 have the roles indicated in Scheme 1. The value for k_2 for the reaction of the ethyl ester is calculated from $1/k_0 = (1/k_2) + (1/k_3)$.

Compound	$(K_m)_2$ (M)	k_2	k_3	k_0
N-Benzoyl-L-tyrosine amide	$2{\cdot}5 \times 10^{-3}$	0·24	300	0·24
N-Benzoyl-L-tyrosine ethyl ester	$1{\cdot}2 \times 10^{-4}$	600	300	200
N-Benzyloxycarbonyl-L-tyrosine nitrophenyl ester	$3{\cdot}2 \times 10^{-5}$	>1000	300	300

kinetics is observed as in our enzyme experiments. From studies of this model system under conditions when reaction half-times from 1 to 10 msec. were observed we could draw the conclusion that for reactions with half-times of 10 msec. or more at least 90% of the course of the reaction can be observed. However, for reactions with half-times of 1 msec. only about 10% of the reaction could be observed, which means that three half-times must have passed between mixing and start of observation.

DISCUSSION

The results presented in Figs. 1 and 2 show clearly that during the reaction of chymotrypsin with CTN there occurs an initial rapid liberation of 1 mole of *p*-nitrophenol/mole of chymotrypsin and a slower subsequent steady-state liberation of *p*-nitrophenol. This sequence of events is similar to that observed for the chymotrypsin-catalysed hydrolysis of *p*-nitrophenyl acetate, except that both the steps characterized by the rate constants k_2 and k_3 (see Scheme 1) are faster by a factor of at least 10^3 for the tyrosine derivative. It is shown that within the time resolution of the method used the initial formation of compound II with CTN is instantaneous. Tests with model systems of the stopped-flow arrangement used here for mixing and observing reactants have shown that the reaction mixture in the observation chamber is about 3 msec. old. The formation of compound II is essentially complete within that time interval and it can be stated therefore that for the chymotrypsin–CTN reaction $k_2 > 1000$ sec.$^{-1}$. The rate constant k_3 of the decomposition of the *N*-benzyloxycarbonyl-L-tyrosine–chymotrypsin compound is given by the steady-state rate k_0 of the overall enzyme reaction for which it is rate-determining. It has been shown that under optimum conditions at 25° $k_0 = 300$ sec.$^{-1}$. This value can be compared directly with the turnover number for chymotrypsin with benzoyl L-tyrosine amide $k_0 = 0{\cdot}24$ sec.$^{-1}$ (MacAllister, 1949) and benzoyl L-tyrosine ethyl ester $k_0 = 200$ (Gutfreund & Sturtevant, 1956*b*).We have suggested (Gutfreund & Sturtevant, 1956*b*) that for the hydrolysis of the amide k_2 is rate-determining, whereas for the hydrolysis of the ethyl ester the two steps characterized by k_2 and k_3 jointly control the overall rate. It can be shown that a comparison of the reactions of the three substrates mentioned above (benzoyl L-tyrosine ethyl ester, benzoyl L-tyrosine amide and CTN) is fully consistent with and gives support to Scheme 1 as a description of chymotrypsin-catalysed hydrolysis reactions. The major difference in the reactions of chymotrypsin with the three substrates is in the rate of the second step, which involves a nucleophilic attack by the catalytic groups of the enzyme on the carbonyl group of the substrate. A comparison of the non-enzymic hydrolysis by nucleophilic reagents shows that aliphatic esters of carboxylic acids are much more rapidly hydrolysed than amides and that nitrophenyl esters are much more rapidly hydrolysed than aliphatic esters (Bender, Ginger & Kemp, 1954; Bender & Turnquest, 1955, 1957*a*, *b*).

Our conclusion about the relative rates for the three substrates of the enzymic step characterized by k_2 (see Table 1) is in accord with the expected chemical reactivities of the different tyrosine compounds. The final step of the enzyme-catalysed hydrolysis reaction, which is characterized by k_3, must be nearly identical for the three tyrosine substrates, since compound II is nearly identical in the three cases.

A comparison of the K_m values for the three types of substrates is also instructive. Gutfreund & Sturtevant (1956*b*) have pointed out that K_m for the overall reaction, i.e. $(K_m)_2$, is in the following relation to $(K_m)_1$:

$$(K_m)_2 = (K_m)_1 \frac{k_3}{k_2 + k_3},$$

where

$$(K_m)_1 = \frac{k_{-1} + k_2}{k_1},$$

$(K_m)_1$ determines the concentration of compound I and $(K_m)_2$ determines the concentration of compound II. As can be seen in Table 1, the faster the rate of formation of compound II, the smaller the value for K_m for the overall reaction.

In this way we have been able to show that tyrosine substrates of chymotrypsin form distinct intermediate compounds with the enzyme. It is only to be regretted that the formation of this compound is so rapid that it has so far not been possible to study the effect of hydrogen ions and changes of other conditions on the rate of its formation. We cannot therefore give a definite answer to the question whether a serine hydroxyl group is acylated by the tyrosyl residue. It is, however, very suggestive that an imidazole-catalysed acylation of the enzyme as well as an imidazole-catalysed hydrolysis of an acyl–enzyme compound occurs in the manner suggested by Gutfreund & Sturtevant (1956*b*) for the hydrolysis of *p*-nitrophenyl acetate. Martin *et al.* (1959) have shown that the overall rate of chymotrypsin-catalysed hydrolysis of CTN has a pH profile corresponding to the involvement of a histidine imidazole residue in the reaction. It is clear from our results that this corresponds to the catalytic role of a free imidazole group in the decomposition of the acyl–enzyme compound and is evidence for a group other than the imidazole group of the catalytic site being responsible for combining the tyrosyl group with the enzyme.

SUMMARY

1. The reactions between chymotrypsin and the *p*-nitrophenyl ester of *N*-benzyloxycarbonyl L-tyrosine have been studied by a fast-reaction photometric method.

2. It has been shown that an *N*-benzyloxycarbonyl-L-tyrosine–chymotrypsin compound is formed as an intermediate in the enzymic hydrolysis of this substrate. The formation of this intermediate compound is so fast ($k > 1000$ sec.$^{-1}$) that its kinetics could not be studied.

3. A comparison of the reaction parameters of the chymotrypsin-catalysed hydrolysis of the amide, ethyl ester and *p*-nitrophenyl ester of tyrosine gives further support to a three-step reaction scheme for enzymic hydrolysis, which has been previously proposed.

We are most grateful to Dr C. J. Martin for a sample of the *p*-nitrophenyl ester of *N*-benzyloxycarbonyl-L-tyrosine as well as for some helpful advice.

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Oxidation of Serotonin and 5-Hydroxyindoles during the Denaturation of Oxyhaemoglobin

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Stacey (1956) reported that serotonin (5-hydroxytryptamine) disappears when it is incubated with washed erythrocytes. In a study of the transport of serotonin into erythrocytes we found no serotonin inside the cells, and it seemed possible that the serotonin was being destroyed by a factor in the erythrocytes. This paper explains why serotonin appeared to be destroyed by erythrocytes. A preliminary report of this work has already appeared (Ling & Blum, 1958).

EXPERIMENTAL AND RESULTS

Blood (about 15 ml.) was obtained from two rats lightly anaesthetized with ether which were bled from the neck into a polyethylene beaker containing 10 ml. of 0·85% NaCl with either 0·1 g. of ethylenediaminetetra-acetic acid or 6 mg. of heparin. The erythrocytes were then centrifuged for 3 min. at 2000 *g* and the supernatant fluid was decanted. The cells were resuspended in 47–50 ml. of 0·12 M-NaCl–0·04 M-Na_2HPO_4 buffer, pH 7·4. The cells were washed three times in about 40 ml. of this buffer. Blood obtained from other species was treated in the same way. When necessary, the washed erythrocytes were haemolysed by adding 2–5 vol. of water. Crystalline haemoglobin was prepared from dog blood by the procedure of Drabkin (1946).

The creatinine sulphate salt of serotonin, bufotenin (*NN*-dimethylserotonin) mono-oxalate, tryptamine hydrochloride, indol-3-ylacetic acid and 5-hydroxyindol-3-ylacetic acid (5-HIAA) were purchased from the California Biochemical Foundation. Crystalline egg albumin, serum globulin, cytochrome *c*, glutathione and 5-hydroxytryptophan were purchased from the Nutritional Biochemical Corp. Catalase was purchased from Worthington Biochemicals Inc., haemin from Eastman Organic Chemicals

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Dept. and iproniazid (*N-iso*nicotinoyl-*N'-iso*propylhydrazine) from Hoffman-La Roche and Co. All other chemicals were reagent-grade.

Serotonin and other 5-hydroxyindoles were assayed spectrophotofluorimetrically in an Aminco–Bowman spectrophotofluorimeter. Deproteinization of samples to be assayed was accomplished with trichloroacetic acid. A sample (2 ml.) of the deproteinized filtrate was mixed with 1 ml. of 9 N-HCl in a 1 cm. square cuvette and the fluorescence was measured with an exciting wavelength (λ_a) of 300 mμ and a fluorescent wavelength (λ_f) of 555 mμ (Udenfriend, Bogdanski & Weissbach, 1955). Since trichloroacetic acid reduces the fluorescence of some 5-hydroxyindoles, standard solutions contained the same final concentration of trichloroacetic acid. In most of the experiments reported here the extinction at 300 mμ of the solutions whose fluorescence was measured was less than 0·4, a range in which fluorescence increases linearly with increasing serotonin concentration. Serotonin was also assayed colorimetrically by the procedure of Udenfriend, Weissbach & Clark (1955).

Preliminary experiments with washed rat erythrocytes confirmed Stacey's (1956) observation that when serotonin was added to a suspension of these cells the serotonin rapidly disappeared from the external solution. It was then found that serotonin also disappeared from solution when haemolysates were used, and it was noted that the deproteinized filtrates were often pink. Since it was possible that erythrocyte 'ghosts' were the active agents, 'ghost'-free haemolysates were prepared. It was found that the activity was unaffected by the removal of the 'ghosts' and thus the presence of an active protein was indicated. The results shown in Table 1 suggested that the reaction might be non-enzymic, since the amount of serotonin lost did not depend on time (for times greater than about 20 sec.), and since the amount of serotonin lost was not simply related to the amount of oxyhaemoglobin added (Fig. 1, curve *B*). When the haemolysate was heated at 60° for 20 min. there was a gradual loss of the ability to destroy serotonin. Heating for 2–3 min. at 80° caused a brown precipitate to form and resulted in a 70 % loss of serotonin-destroying ability. There was little effect of pH between 4·0 and 10·0 on the ability of a haemolysate to destroy serotonin (Table 2), but at pH 3·0 where a brownish precipitate formed, serotonin-destroying activity was lost. The apparently non-enzymic nature of the reaction and the range of heat and of pH sensitivity suggested that the active protein was haemoglobin.

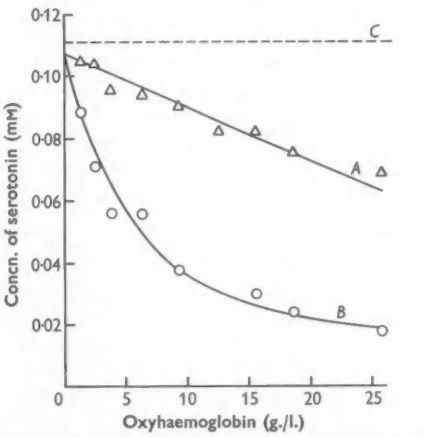

Fig. 1. Destruction of serotonin by oxyhaemoglobin. Curve *B* represents a reaction mixture containing the indicated amounts of crystalline dog oxyhaemoglobin, to which was added serotonin to make a final concentration of 0·11 mM, followed by trichloroacetic acid to give a final concentration of 4·6 % (w/v). Conditions for curve *A* were the same except that the trichloroacetic acid was added to the oxyhaemoglobin before the addition of serotonin. Curve *C* is the serotonin concentration that would have been found if no serotonin had been destroyed. Measurements were made by the colorimetric method of Udenfriend, Weissbach & Clark (1955).

Table 1. *Effect of time of incubation on the reaction of serotonin with different amounts of haemolysed erythrocytes*

Washed erythrocytes from 6·5 ml. of rat blood were suspended in 20 ml. of buffer. This was diluted 1:10 with water to make the haemolysate. To the indicated amounts of haemolysate at room temperatures was added 0·364 μmole of serotonin. Samples were withdrawn at the times shown and were deproteinized with trichloroacetic acid (final concn. 0·306 M) and assayed spectrophotofluorimetrically.

	Serotonin found					
Vol. of haemolysate (ml.)	... 0·5		1·0		3·0	
Time (sec.)	(μmole)	(% loss)	(μmole)	(% loss)	(μmole)	(% loss)
20	0·050	15·0	0·045	23·5	0·032	45·6
100	0·056	4·8	0·048	18·4	0·031	47·2
170	0·055	6·5	0·049	16·7	0·033	43·8
250	0·054	8·2	0·045	23·5	0·033	43·8
700	0·055	6·5	0·049	16·7	0·032	45·6

Table 2. *Effect of* pH *on the reaction of serotonin with haemolysed erythrocytes*

Washed erythrocytes from 15 ml. of rat blood were haemolysed with water to a final volume of 50 ml. To 1 ml. of haemolysate were added 2 ml. of 0·2 M-buffer of composition and pH shown in the table, and then 5 ml. of the appropriate concentration of serotonin in water. The resulting mixture was incubated for 5 min. at room temperature, and then 2 ml. of 10 % (w/v) trichloroacetic acid was added. The serotonin was then assayed spectrophotofluorimetrically. Appropriate standards and blanks were also prepared. Sodium acetate and sodium tartrate were employed, and the pH was adjusted with NaOH or HCl as necessary. Tris, 2-amino-2-hydroxymethylpropane-1:3-diol.

Serotonin in incubation mixture (μmole)	Serotonin loss (%)				
	Acetate pH 4·0	Acetate pH 6·0	Tris pH 8·0	Tris pH 10·0	Tartrate pH 3·0
0·0036	94	99	99	97	—
0·0073	93	99	99	98	19
0·0127	92	96	99	98	—
0·0182	91	97	97	97	12
0·0364	73	89	90	90	—
0·0910	39	62	65	70	2
0·1820	37	40	48	50	—

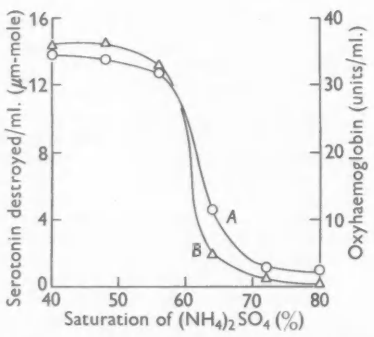

Fig. 2. Ammonium sulphate fractionation of active protein. The washed erythrocytes from 13 ml. of rat blood were haemolysed with water to a final volume of 50 ml. Samples (5 ml.) were then adjusted to 25 ml. by the addition of appropriate amounts of saturated $(NH_4)_2SO_4$ and of water. The tubes were kept at 4° for 3 hr. and then centrifuged for 10 min. at 12 800 *g*. To the supernatant fluid serotonin was added to make the final concentration 14·2 μm-moles/ml. After a few minutes trichloroacetic acid (final concn. 0·3 M) was added and serotonin was assayed spectrophotofluorimetrically. Portions of the supernatant fluid were diluted with appropriate amounts of water and their extinction was measured in an Evelyn colorimeter. An oxyhaemoglobin unit is defined as the quantity of oxyhaemoglobin in aqueous solution which has an extinction of unity in this instrument, with a 420 mμ filter. Curve *A* shows the amount of serotonin destroyed, and curve *B* is proportional to the oxyhaemoglobin concentration.

Nature of the active protein

An ammonium sulphate-fractionation procedure was employed to purify the active protein. As is evident from Fig. 2, the serotonin-destroying activity (curve *A*) was precipitated under the same conditions as the oxyhaemoglobin (curve *B*). Since rat oxyhaemoglobin is difficult to redissolve after crystallization, crystalline dog oxyhaemoglobin was tested and found to destroy serotonin (Fig. 3). The results from this and other experiments show that 95 % loss of serotonin occurs when the molar ratio of oxyhaemoglobin to serotonin is 5 to 1 or greater, and that 30 % loss occurs when the ratio of oxyhaemoglobin to serotonin is 1 to 5 or less. We have been unable to obtain precise stoicheiometric relations for several reasons. Experiments with a fixed amount of oxyhaemoglobin and varying serotonin (Fig. 3) have a rather sharp break, suggesting a stoicheiometric relation between the amount of oxyhaemoglobin and the amount of serotonin which can be destroyed. Experiments with a fixed amount of serotonin and varying amounts of oxyhaemoglobin (Fig. 1, curve *B*), however, do not show a linear relation between the amount of serotonin destroyed and the amount of oxyhaemoglobin present. Furthermore, it is found that at very high concentrations of serotonin

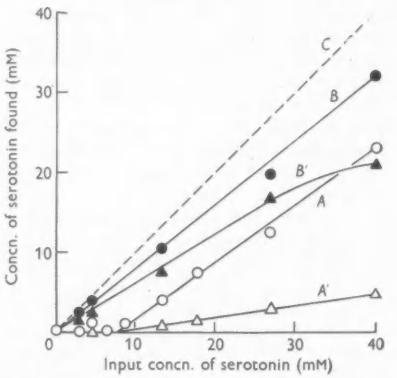

Fig. 3. Destruction of serotonin in the presence of a fixed amount of oxyhaemoglobin. Curves *A* and *A'* (open symbols) represent a reaction mixture containing 12·4 mg. of crystalline dog oxyhaemoglobin/ml., to which was added the indicated amounts of serotonin, followed by trichloroacetic acid to give a final concentration of 4·6 % (w/v). Conditions for *B* and *B'* (filled-in symbols) were the same except that the trichloroacetic acid was added to the oxyhaemoglobin before the addition of serotonin. Curve *C* is a reference curve for 100 % recovery of added serotonin. ○, ●, Measurements obtained colorimetrically; △, ▲, spectrophotofluorimetric measurements of portions of the same reaction mixtures.

Table 3. *Effects of carbon monoxide on the oxidation of serotonin by oxyhaemoglobin*

The components of the reaction mixture were added in the sequence shown, and the serotonin remaining in the filtrate was assayed spectrophotofluorimetrically. Final volume in each tube was 4·5 ml. The final concentrations were: oxyhaemoglobin (O_2Hb), 18·7 mg./ml.; serotonin (S), 0·111 mM; trichloroacetic acid (TCA), 5·6% (w/v); buffer (B), 0·02 M-Na_2HPO_4, pH 8·5; CO was bubbled through the mixture for 2 min.

Tube no.	1	2	3	4	5	6
Sequence of addition of reagents	O_2Hb	O_2Hb	O_2Hb	O_2Hb	O_2Hb	O_2Hb
	TCA	S	CO	B	CO	S
	S	CO	S	CO	TCA	TCA
	CO	TCA	TCA	TCA	B	B
Final concn. of serotonin (mM) ...	0·088	0·079	0·090	0·000	0·000	0·005

Table 4. *Effect of addition of oxygen on the oxidation of serotonin by haemoglobin*

Serotonin (10 μmoles) was added to two solutions, each containing 100 mg. of crystalline dog haemoglobin. The final volume was adjusted to 25 ml. The serotonin was assayed spectrophotofluorimetrically in the presence of 0·113 M-trichloroacetic acid.

Treatment	Time (sec.)	Loss (%)
Bubbling oxygen	270	27
	445	27
	910	28
	2000	29
None	230	27
	480	26
	940	29
	2020	27

(greater than 10 mM) the amount lost appears larger when the deproteinized reaction mixture is assayed spectrophotofluorimetrically (Fig. 3, curves A' and B') than when the same mixture is assayed colorimetrically (Fig. 3, curves A and B). The difference between the two methods at high serotonin concentrations may be an artifact resulting from the high extinctions (greater than 2) at 300 mμ of the solution to be assayed in the photofluorimeter. At lower concentrations both methods give results which agree completely. Another complication arises from the observation that the controls, in which oxyhaemoglobin was denatured by trichloroacetic acid before the addition of serotonin, are still capable of destroying some serotonin (Fig. 1, curve A), though without the production of the characteristic pink product to be described below.

Exposure of oxyhaemoglobin to CO (Table 3) caused an almost complete loss of activity, thus suggesting that the Fe^{2+} ion of the haem is necessary for the destruction of serotonin. The finding that even large concentrations (0·3 M) of KCN do not greatly reduce the serotonin-destroying activity of a given amount of oxyhaemoglobin is further evidence that the active protein is ferrohaemoglobin. Incubation of serotonin with $FeCl_3$ instead of oxyhaemoglobin caused a very slow destruction of serotonin, and hence traces of it could not be responsible for the activity of oxyhaemoglobin. No activity was detected with solutions of crystalline egg albumin (0·19%), bovine serum globulin (0·19%), catalase (0·38%) or cytochrome *c* (0·14%). Haemolysates of human, turkey, pig, cow, chicken, rabbit and cat erythrocytes were as active as rat-erythrocyte haemolysates.

When O_2 was bubbled through a reaction mixture there was no increase in the destruction of serotonin (Table 4), but when O_2 was removed from oxyhaemoglobin (and from the serotonin and trichloroacetic acid solutions) there was little or no loss of serotonin (Table 5). Glutathione and $NaHSO_3$ (0·33 M) did not prevent the destruction of serotonin by oxyhaemoglobin. Ascorbic acid, however, largely prevented the destruction of serotonin (Table 6).

No change was found in the light absorption of oxyhaemoglobin after the addition of serotonin. Since it seemed possible that the ability of oxyhaemoglobin to destroy serotonin was due to its well-known pseudo-peroxidase activity (Willstätter & Pollinger, 1923), a solution of crystalline dog oxyhaemoglobin was pre-incubated with 5 mg. of catalase for 10 min. before the addition of serotonin. Upon deproteinizing with trichloroacetic acid and assaying, there was found little or no difference in the pink colour or in the amount of serotonin destroyed as compared with a control not treated with catalase. The possibility that organic peroxides associated with the crystalline oxyhaemoglobin were responsible for the activity was further checked by incubating quinol with the oxyhaemoglobin. There was no colour in the deproteinized filtrate, although the addition of H_2O_2 to a sample of quinol rapidly yielded a yellowish brown.

Carbon monoxide protects serotonin against oxidation by oxyhaemoglobin even when the CO is added after the serotonin and oxyhaemoglobin have been in contact (Table 3). Thus the oxidation of serotonin does not occur until the denaturation of oxyhaemoglobin takes place. The oxidation occurs whether the denaturation is caused by butanol or by trichloroacetic acid. Serotonin is oxidized more easily than ascorbic acid and much more easily than glutathione. Unlike ascorbic acid and glutathione

Table 5. *Effect of haemoglobin and of oxyhaemoglobin on serotonin*

The components were added in the sequence shown. Deoxygenation was achieved by evacuation of the system with an oil pump followed by flushing with N_2 and re-evacuation. The final concentrations were (abbreviations as in Table 3): Hb or O_2Hb, 13·4 mg./ml.; S, 0·111 mM; TCA, 5·6% (w/v). Total volume was 4·5 ml. Serotonin in the filtrate was assayed spectrophotofluorimetrically.

	No O_2		O_2	
Sequence of addition of reagents	Hb S TCA	Hb TCA S	O_2Hb S TCA	O_2Hb TCA S
Final concn. of serotonin (mM) ...	0·096	0·096	0·014	0·100

Table 6. *Effect of ascorbic acid on the reaction of serotonin with haemolysed erythrocytes*

A dialysed haemolysate (3 ml.) of dog erythrocytes, containing 46·8 mg. of oxyhaemoglobin/ml. was placed in each tube, followed by 10 ml. of ascorbic acid of the desired concentration dissolved in 0·2M-Na_2HPO_4, pH 7·4, and then by 0·5 μmole of serotonin in 1 ml. of water. A portion (2 ml.) of 25% (w/v) trichloroacetic acid was added and serotonin in the filtrate was measured at λ_a 300 mμ, λ_f 360 mμ, without the addition of 3N-HCl.

Tube no.	Ascorbic acid (mM)	Serotonin (% loss)	Colour
1	7·14	40	None
2	4·28	40	None
3	2·86	40	None
4	1·43	42	Barely pink
5	0·71	50	Light pink
6	0·36	61	
7	0·29	62	
8	0·21	74	
9	0·14	77	Increasing pink
10	0·07	82	
11	0·04	89	
12	0·00	94	

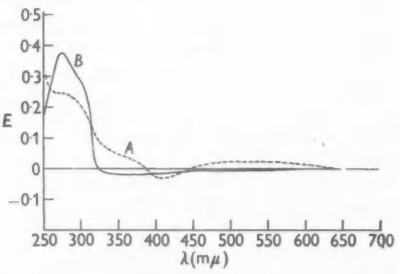

Fig. 4. Absorption of serotonin and of serotonin oxidation product(s). Washed erythrocytes from 14·5 ml. of heparinized rat blood were haemolysed with water to a final volume of 50 ml. Curve *A* is the spectrum of the filtrate of 3 ml. of haemolysate, 5 ml. of 0·185 mM-serotonin and 3 ml. of 10% (w/v) trichloroacetic acid, mixed in that sequence. Curve *B* is the spectrum obtained when the same quantities were mixed in the sequence haemolysate, trichloroacetic acid, serotonin. Extinction was measured in a Beckman DU spectrophotometer in 1 cm. cells, with a blank in which 5 ml. of water replaced the 5 ml. of serotonin. In this reaction there was a 74% loss of the added serotonin (spectrophotofluorimetric assay). Curve *A* has not been corrected for the remaining serotonin. If this were done that part of curve *A* in the region from 250–300 mμ would have a small decrease in extinction, and there would be no discernible change at wavelengths greater than 300 mμ.

(Numata, 1940), serotonin is slowly destroyed in the presence of denatured oxyhaemoglobin (Fig. 1, curve *A*).

Specificity of the reaction

Several analogues of serotonin were incubated with haemolysates of rat erythrocytes. Bufotenin (5-hydroxy-*NN*-dimethyltryptamine), 5-hydroxytryptophan and 5-HIAA all reacted as measured by fluorescence loss at λ_a 300, λ_f 555 mμ in 3N-HCl. The ultraviolet spectra were similar to that shown in Fig. 4 for the oxidation product of serotonin. Bufotenin gave a product with the same pink colour as did serotonin, but 5-hydroxytryptophan and 5-HIAA yielded an orange–pink product. Tryptamine and indole-3-acetic acid were not affected by denaturing oxyhaemoglobin.

Nature of the reaction product(s)

Owing to the instability of the product, several attempts to isolate it were unsuccessful [e.g. Amberlite XE-64 resin in the ammonium form, Celite, paper chromatography (Rodnight, 1956) and paper electrophoresis]. The product is characterized by a loss of fluorescence both in 3N-HCl at λ_a 300, λ_f 555 mμ and at pH 1·0–7·0 at λ_a 300, λ_f 360. No new fluorescent peaks were observed. There is also a loss of 5-hydroxyindole material as measured by the colorimetric method of Udenfriend, Weissbach & Clark (1955). The absorption spectrum (Fig. 4) changes both in the ultraviolet and in the visible. The pink colour fades in about half an hour at pH 5·0 and lasts for half a day in trichloroacetic acid. It changes to yellow instantly upon the addition of excess of NaOH, and disappears in a few minutes in 3N-HCl. The pink product of serotonin oxidation was not extracted from the aqueous phase with benzene, chloroform,

light petroleum or carbon tetrachloride, but was extracted into butanol, in which the colour was stable for several days. The product of bufotenin oxidation remained in the aqueous phase with all the above-mentioned solvents including butanol. When a concentrated solution of serotonin was oxidized by concentrated denaturing oxyhaemoglobin, the filtrate was a turbid brownish pink which formed a black precipitate overnight. The addition of ascorbic acid to the pink product causes a rapid loss of colour, but the fluorescence does not return.

The product was tested for possible biological activity on the heart of the clam *Venus mercenaria*, with essentially the bioassay procedure of Twarog & Page (1953). No activity was found for the oxidized products of serotonin, bufotenin, 5-hydroxytryptophan or 5-HIAA.

DISCUSSION

The present results show that during the denaturation of oxyhaemoglobin there is a strong peroxidase-like activity which can attack 5-hydroxyindole compounds, as well as glutathione and ascorbic acid, as was found by Numata (1940). Rodnight (1958) has also noted that under certain conditions oxyhaemoglobin can destroy serotonin and he suggested that the process was similar to the oxidation of ascorbic acid and glutathione by denaturing oxyhaemoglobin. The product of this oxidation may be similar to a compound first observed by Cromartie & Harley-Mason (1957), who found that when oxygen is bubbled through a solution of 5:6-dihydroxy-2:3-dimethylindole, a pink product develops and the ultraviolet-absorption spectrum changes. The absorption spectrum of their product is similar to the spectrum of the oxidized product formed by the action of oxyhaemoglobin undergoing denaturation on serotonin (Fig. 4). It is thus possible that in the reaction with oxyhaemoglobin the 5-hydroxyindoles are first hydroxylated and then further oxidized. The formation of a pink product from serotonin is reminiscent of the oxidation of adrenaline solutions by atmospheric oxygen, and there may be some similarity in the structure of the serotonin oxidation product and compounds like adrenochrome (Harley-Mason, 1950; Beaudet, Debot, Lambot & Toussaint, 1951).

These results, in addition to providing further information on the oxidizing properties of haemoglobin undergoing denaturation, indicate that a system containing oxyhaemoglobin should be saturated with carbon monoxide before attempting to assay for 5-hydroxyindoles.

SUMMARY

1. Oxyhaemoglobin, when it is undergoing denaturation, can oxidize serotonin and several related 5-hydroxyindoles. The reaction is completely inhibited by carbon monoxide and partially inhibited by ascorbic acid.

2. The oxidized product(s) is characterized by a pink colour, a change in the ultraviolet-absorption spectrum and a loss of fluorescence.

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The Isolation and Structure of Actinomycins II and III

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Since the discovery of actinomycin A by Waksman & Woodruff (1940) it has been recognized (Vining & Waksman, 1954) that the actinomycin complexes A, B and X consist of the same components, actinomycins I, II ,III, IV and V, in different proportions and that actinomycin D is essentially pure actinomycin IV. The structure of actinomycin IV (I; A = B = L-proline) has been determined by Bullock & Johnson (1957). Recently, Katz, Pienta & Sivak (1958) have shown that the composition of

actinomycin A varies according to the conditions of growth of the organism, and Katz & Goss (1958, 1959) have studied the effect of adding various amino acids to the culture medium of *Streptomyces antibioticus*. In particular, they found that the addition of sarcosine stimulated the production of actinomycins II and III, which are normally trace components, and that with an optimum concentration (0·05 %) of sarcosine these two actinomycins accounted for about 25 and 35 % respectively of the complex.

This paper describes the properties of actinomycins II and III isolated from this sarcosine-stimulated actinomycin complex.

EXPERIMENTAL

A sample of the actinomycin complex produced by the sarcosine-stimulated organism was provided through the courtesy of Professor Waksman and Dr Katz of the Institute of Microbiology, Rutgers State University.

All melting-point determinations were carried out on the Kofler hot-plate. 'Acid-washed alumina' refers to alumina (type H; P. Spence and Sons, Ltd., Widnes, Lancs) which has been washed for 2 hr. with 10 % (v/v) acetic acid at 70°, with hot water to pH 4–5 and then with methanol, and dried in air.

Paper chromatography of the actinomycin complex produced by the sarcosine-stimulated organism. Circular paper chromatograms were run on disks (24 cm. diam.) of Whatman no. 1 paper in the solvent system di-*n*-butyl ether–tetrachloroethane–10 % (w/v) aq. sodium *o*-cresotinate (2:1:3, by vol.) (Roussos & Vining, 1956), the paper being first impregnated with the aqueous phase. Actinomycins B and D were run on the same paper chromatograms to provide reference components. The crude complex was thus shown to consist of five principal components: I (R_{IV} 0·17), II (R_{IV} 0·45), III (R_{IV} 0·68), IV (R_{IV} 1·00) and V (R_{IV} 1·57) and another actinomycin (R_{IV} 1·28). (R_{IV} indicates the R_F relative to that of actinomycin IV.)

Adsorption chromatography of actinomycin complex. A solution of the crude complex (884 mg.) in benzene–chloroform (3:1, v/v) (50 ml.) was filtered and then brought on to a column (32 cm. × 4·8 cm.) of acid-washed alumina. Elution was commenced with the same solvent mixture, the proportion of chloroform being gradually raised to benzene–chloroform (1:3, v/v). The main orange band was collected in three fractions and elution was then continued with 1 % methanol in chloroform to collect a slower-running minor band in a fourth fraction. Each fraction was concentrated and examined by paper chromatography by the method described above. The first 600 ml. of eluate contained no actinomycins. Fraction (i) (250 ml.) consisted mainly of fast-running trace components which were more conveniently examined by paper chromatography in the solvent system di-*n*-butyl ether–tetrachloroethane–10 % (w/v) aq. sodium *o*-cresotinate (4:1:5, by vol.). Comparison with the actinomycin C complex (R_{IV} values: C_1, 1·00; C_2, 1·83; C_3, 3·17) on the same circular chromatogram indicated that one of these trace components corresponded with C_2 (Brockmann & Pfennig, 1952). Fraction (ii) (700 ml.) consisted principally of actinomycins III, IV and V, and fraction (iii) (700 ml.) consisted almost entirely of actinomycins II and III. Fraction (iv) (800 ml.) consisted mainly of actinomycin I. The solvent was removed from fraction (ii) and the residue (280 mg.) was subjected to chromatography on a column (25 cm. × 1·9 cm.) of acid-washed alumina, the elution being commenced with benzene–chloroform (3:1, v/v) and gradually proceeding to benzene–chloroform (2:3, v/v), at which stage the main orange band had been collected. Five fractions were collected. Fraction (*a*) (300 ml.) contained actinomycin IV plus higher trace-components. Fraction (*b*) (100 ml.) was similar to fraction (*a*). Fraction (*c*) (75 ml.) contained actinomycin IV plus a trace of actinomycin III. Fraction (*d*) (75 ml.) contained actinomycins III and IV. Fraction (*e*) (300 ml.) contained actinomycins II, III, IV and the component of R_{IV} 1·28. Fractions (*c*), (*d*) and (*e*) were combined with fraction (iii) from the first column and the solvent was removed, leaving a red, semi-crystalline residue (377 mg.).

Preparative paper chromatography of the concentrated actinomycin complex. The mixture of actinomycins (377 mg.) obtained by adsorption chromatography was dissolved in acetone (25 ml.) and this solution (15 mg. of solid per sheet) was applied in horizontal streaks to square sheets (12 in. × 12 in.) of Whatman 3 MM paper which had been previously impregnated with the aqueous phase of the solvent system di-*n*-butyl ether–tetrachloroethane–10 % aq. sodium *o*-cresotinate (2:1:3, by vol.). The mixture was separated on each sheet by the ascending chromatographic technique. The papers were dried in air and the coloured zones were cut out. Corresponding zones were combined and extracted in a Soxhlet apparatus with acetone (250 ml.).

Actinomycin III. The acetone extracts were evaporated and the residue, which was contaminated with salts, was shaken with water (100 ml.) and chloroform (100 ml.) and the layers were separated. The aqueous phase was washed with chloroform (3 × 25 ml., then 6 × 10 ml.); the chloroform extracts were combined, washed with water (3 × 100 ml.) and dried and the solvent was removed. The residue was dissolved in benzene–chloroform (2 ml.; 1:1, v/v) and chromatographed on a column (10 cm. × 1·0 cm.) of acid-washed alumina, with benzene–chloroform (1:1, v/v) as eluting solvent. The single orange band was collected and the solvent removed, leaving a red semi-crystalline residue (102 mg.) which crystallized as prisms, m.p. 237–238°, $[\alpha]_D^{19}$ – 205° in chloroform (*c*, 0·22), from ethanol containing a little chloroform (Found, in a sample dried *in vacuo* at 78°: C, 58·4; H, 7·2; N, 13·1. $C_{60}H_{84}O_{16}N_{12}$ requires C, 58·6; H, 6·9; N, 13·7 %). Light-absorption in chloroform–ethanol (1:10): max. at 240 (log ϵ, 4·54), 430 (log ϵ, 4·38) and 450 mμ (log ϵ, 4·41). The infrared absorption (KBr disk) showed maxima at 781, 953, 1080, 1100, 1131, 1202, 1230, 1272, 1303, 1322, 1362, 1380, 1410, 1489, 1515, 1588, 1642, 1664, 1758, 2965 and 3430 cm.$^{-1}$. Circular paper chromatography of actinomycin III indicated the presence of only one component, R_{IV} 0·68.

Actinomycin II. Actinomycin II was obtained by the same method as actinomycin III, from the appropriate extracts of the preparative paper chromatograms. It crystallized from acetone–carbon disulphide as plates, m.p. 215–216°, $[\alpha]_D^{17}$ – 157° in chloroform (*c*, 0·24) (Found, in a sample dried *in vacuo* at 100°: C, 58·0; H, 6·83; N, 13·8. $C_{58}H_{82}O_{16}N_{12}$ requires C, 57·9; H, 6·9; N, 14·0 %). Light-absorption in chloroform–ethanol (1:10): max. at

237 (log ϵ, 4·55), 429 (log ϵ, 4·33) and 447 mμ (log ϵ, 4·37). The infrared spectrum (KBr disk) showed maxima at 1079, 1131, 1200, 1230, 1319, 1401, 1489, 1517, 1587, 1660, 1750, 2330, 2351, 2930, 2960 and 3390 cm.$^{-1}$. Circular paper chromatography of actinomycin II indicated the presence of one component only, R_{IV} 0·45.

Amino acid content of actinomycin III. Actinomycin III (10·19 mg.) was dissolved in 6N-HCl (2 ml.) in a sealed tube and the solution was heated at 100° for 46 hr., then taken to dryness *in vacuo* and twice dissolved in water (1 ml.), evaporated to dryness, re-dissolved in water and transferred quantitatively through a filter plug into a standard flask and diluted to 5 ml. For comparison, actinomycin D (12·39 mg.) was treated in the same way. The hydrolysates were run on two-dimensional paper chromatograms in (i) phenol–water (500 g. of phenol to 112 ml. of water) and (ii) butan-1-ol–acetic acid–water (4:1:5, by vol.). Actinomycin III gave the following spots on treatment with ninhydrin (R_F values in the butanol solvent first; colour purple unless otherwise stated): sarcosine (0·17, 0·73); threonine (0·19, 0·44); proline (0·25, 0·85, yellow); valine (0·39, 0·72) and *N*-methylvaline (0·42, 0·93). Descending paper chromatograms of the actinomycin III and IV hydrolysates were run in butan-2-ol–aq. 3% NH_3. The chromatograms were run for 4 days, the solvent being allowed to drip from the serrated edge of the paper. The papers were dried in air and treated first with isatin (0·2% in acetone) and heated at 100°, and then with ninhydrin (0·2% in acetone) and again heated at 100°. The various spots in the two hydrolysates corresponded exactly, the distances run being as follows (purple unless otherwise stated): threonine (7·7 cm.); sarcosine (9·6 cm.); proline (11·7 cm., blue); valine (17·5 cm.) and *N*-methylvaline (24·0 cm.).

The proline content of actinomycin III was determined as follows: paper chromatograms to which measured quantities (microsyringe) of actinomycins IV and III hydrolysates had been applied were run in (i) butan-2-ol–aq. 3% NH_3 and (ii) butanol–acetic acid–water (4:1:5, by vol.). The papers were dried, dipped in a solution of isatin (0·2% in butanol containing 4% acetic acid) and kept in the dark at room temperature for 24 hr. The blue spots were scanned at intervals of 0·1 in. along the mid-line with a reflexion densitometer, and curves of densitometer reading against distance were drawn. The values obtained for the proline content of actinomycin III, calculated by comparison of the areas under the curves with those obtained for actinomycin IV, were (i) 1·0 mole and (ii) 1·1 moles.

The method of Krishnamurthy & Swaminathan (1955) was applied to circular paper chromatograms of actinomycins IV and III hydrolysates run in the solvent system butanol–phenol–acetic acid–water (30:10:8:40, by vol.) (R_F values: threonine, 0·23; sarcosine, 0·29; proline, 0·41; valine, 0·49; *N*-methylvaline, 0·59). Comparison of the two hydrolysates gave the following molar ratios for actinomycin III: valine:*N*-methylvaline:sarcosine, 2·0:2·1:2·9 (molecular weights: actinomycin IV, 1255; actinomycin III, 1229; actinomycin II, 1203). Threonine analyses were unsatisfactory because of decomposition of this amino acid during hydrolysis.

Amino acid content of actinomycin II. Actinomycin II (6·46 mg.) was dissolved in 6N-HCl (1 ml.) and the solution was heated in a sealed tube at 100° for 46 hr., and then evaporated and made up to 5 ml. by the procedure used for actinomycins III and IV. A two-dimensional paper chromatogram of the hydrolysate in the same solvent systems as those used previously gave the following spots (all purple) after ninhydrin treatment (R_F in the butanol solvent first): sarcosine (0·24, 0·73); threonine (0·23, 0·43); valine (0·41, 0·72); *N*-methylvaline (0·49, 0·91). Comparison of the hydrolysate with that of actinomycin III by descending paper chromatography in butan-2-ol-aq. 3% NH_3 showed the two hydrolysates to be identical, except that the proline spot was absent from the actinomycin II hydrolysate. The method of Krishnamurthy & Swaminathan (1955) was applied as before to actinomycin II hydrolysate. Comparison with actinomycin IV hydrolysate gave the following molar ratios for actinomycin II: valine: *N*-methylvaline:sarcosine, 2·0:1·9:3·7.

Oxidation of actinomycin III. Actinomycin III (15 mg.) was dissolved in methanol (1·5 ml.) containing chloroform (0·1 ml.), and aq. 30% H_2O_2 (0·2 ml.) was added, followed by aq. 2% NaOH (1 ml.) and water (0·5 ml.). After 1 hr. at room temperature aq. 30% H_2O_2 (0·2 ml.) was again added. After a further hour at room temperature the solution was adjusted to pH 3–4 with HCl, diluted with water (35 ml.) and extracted with butan-1-ol (3 × 10 ml.). The combined butanol extracts were washed with water (10 ml.) and evaporated to dryness, leaving a pale-yellow amorphous solid. Paper chromatography of the latter in propan-2-ol–aq. NH_3 (sp.gr. 0·880)–water (7:1:2, by vol.) gave two spots; one, fluorescent in u.v. light, had R_F 0·51. The oxidation product was streaked on a sheet (18 in. × 5 in.) of Whatman 3MM paper and run by the descending method in the same solvent system. The relevant areas of the paper were cut out and extracted with ethanol, the ethanol was removed *in vacuo* and the two residues were hydrolysed in sealed tubes in 6N-NCl at 117° for 7 hr. Paper-chromatographic examination of the hydrolysates indicated that both fractions contained the same five amino acids as did actinomycin III itself.

DISCUSSION

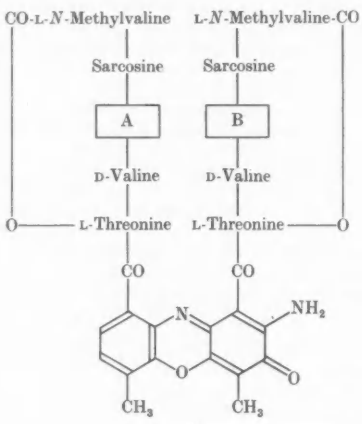
Chromatography of the complex on columns of acid-washed alumina gave only a partial separation of the various components; a final separation was achieved by preparative paper chromatography with the solvent system (Roussos & Vining, 1956) di-*n*-butyl ether–tetrachloroethane–10% sodium *o*-cresotinate.

Some of the physical properties of the pure crystalline actinomycins are given in Table 1.

Table 1. *Physical properties of actinomycins II, III and IV*

Actinomycin	Crystalline form	M.p.	$[\alpha]_D^{19}$ ($CHCl_3$)	$\lambda_{max.}$ (ethanol)
II	Plates	215–216°	−157°	237, 447
III	Prisms	237–238°	−205°	240, 450
IV	Prisms	235·5–236·5°	−337°	242, 444

Paper-chromatographic examination of the amino acids released after acid hydrolysis of the actinomycins showed that actinomycin III contains the same five amino acids (threonine, valine, proline, sarcosine and *N*-methylvaline) as does actinomycin IV, but that actinomycin II lacked proline. The relative proportions of the various amino acids present in the two hydrolysates was determined by paper-chromatographic methods, by the method of Krishnamurthy & Swaminathan (1955) and by taking densitometer readings on paper chromatograms carrying also an actinomycin IV hydrolysate as a standard, after development of the amino acid spots with ninhydrin. The results showed that actinomycin III contains three sarcosine residues and one proline residue and that actinomysin II contains four sarcosine residues and no proline, whereas actinomycin IV contains two sarcosine residues and two proline residues. Actinomycins IV, III and II thus constitute a series in which proline and sarcosine are interchangeable. Brockmann & Manegold (1958) have shown that actinomycins I ('$X_{0\beta}$') and V ('X_2') differ from IV ('X_1') in that the proline has been replaced partially by 4-hydroxyproline and 4-oxoproline respectively; otherwise the sequence of amino acids in the peptide chains was the same as in those of the actinomycins of the C series (where the D-valine of actinomycin D is replaced by D-*allo*isoleucine). A tentative structure for the actinomycins II and III is therefore shown in (I)



(I)

Actinomycin II: A = B = Sarcosine

Actinomycin III: A = Sarcosine; B = L-proline and/or A = L-proline; B = Sarcosine

Actinomycin IV: A = B = L-Proline

Bossi, Hütter, Keller-Schierlein, Neipp & Zahner (1958) have shown that 'actinomycin Z' contains *N*-methylalanine and no proline, but the crude product contained six components and information is not yet available on the detailed structures.

Actinomycin III may be a mixture of two isomers formed by random oxidative coupling (Johnson, 1956) of the two 3-hydroxy-4-methylanthraniloyl peptides, i.e. II-type and IV-type peptides. These isomers would be difficult to separate by chromatography, but a possible means of detecting their presence would be by oxidative fission and subsequent hydrolysis (Bullock & Johnson, 1957). Paper-chromatographic separation and subsequent hydrolysis of the two pentapeptides obtained by a small-scale oxidation of actinomycin III indicated that all five amino acids were present in both peptides; had only one possible form of actinomycin III been present then one of the peptides would have lacked proline.

SUMMARY

1. A sarcosine-stimulated actinomycin complex has been resolved into its components by preparative paper chromatography. Actinomycins II and III have been isolated and their physical properties are described.

2. The amino acid contents of these two actinomycins have been determined and tentative structures are proposed.

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Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

ENZYMIC AND CHEMICAL PROPERTIES OF THE CRYSTALLINE ENZYME

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Bach, Dixon & Zerfas (1946) found that cytochrome b_2 (Bach, Dixon & Keilin, 1942) was an essential part of the dehydrogenase system of baker's yeast which catalysed the reduction of methylene blue by lactate. Appleby & Morton (1954) reported that purified crystalline lactic dehydrogenase of yeast was a chromoprotein having both protohaem and riboflavin phosphate as essential prosthetic groups and was identical with cytochrome b_2.

The procedure for purification and crystallization of the enzyme has been published (Appleby & Morton, 1959). Some enzymic and chemical properties of the crystalline material are described here. This work was described at the meeting of the Australian and New Zealand Association for the Advancement of Science in Melbourne, 1955, and some preliminary accounts have been published (Appleby & Morton, 1954; Morton, 1955).

MATERIALS

Crystalline yeast lactic dehydrogenase (*cytochrome* b_2). This was prepared as described by Appleby & Morton (1959). Unless otherwise stated, the material was recrystallized at least once, and sometimes twice, from a solution of the combined first crystals from a number of preparations.

Hydroxy acids. DL-α-Hydroxy-*n*-butyric acid (Bios Laboratories Ltd.), DL-β-hydroxy-*n*-butyric acid (British Drug Houses Ltd.) and α-hydroxy-*iso*-butyric acid (Eastman Organic Chemicals Ltd.), at approx. molar concentration, were hydrolysed at 100° for 1 hr. and then adjusted to about pH 7·0 with 5 N-NaOH and diluted to 0·5 M concentration. Glycollic acid (British Drug Houses Ltd.) was treated similarly except that hydrolysis was omitted. DL-Lactic acid was prepared as described previously (Appleby & Morton, 1959).

Pyridine. Analytical-grade reagent (British Drug Houses Ltd.) was redistilled, the first and last portions of the distillate being discarded.

METHODS

Estimation of enzymic activity. The units of activity and the assay procedures have been described by Appleby & Morton (1959). For estimation of the optimum pH for activity, the buffer system of Michaelis (1931) was used with crude enzyme preparations and 0·1 M-sodium pyrophosphate–acetic acid buffer with crystalline enzyme. All reaction mixtures contained 1 ml. of the appropriate buffer in a final volume of 3 ml. The pH values were determined at the conclusion of each experiment with a glass electrode at room temperature.

Molecular extinction coefficient (ε). In this paper, values of ε given for cytochromes refer to the extinction coefficients for molar concentration of protein-bound haem.

Chemical determinations

Protohaem. This was estimated as the pyridine haemochromogen. Pyridine (0·6 ml.) was added to a suitable amount of material (as dried powder, aqueous suspension or solution) in a Thunberg-type cuvette (Appleby & Morton, 1959). After gentle shaking, about 0·75 ml. of 0·2 N-KOH was added and the volume brought to 3 ml. with water. After evacuation, the cuvette was filled with O_2-free N_2 and about 3 mg. of $Na_2S_2O_4$ was added from the side bulb. The absorption spectrum was determined after 15 min. The haemochromogen so prepared from crystalline protohaemin had ϵ $33{\cdot}2 \times 10^3$ at 556·5 mμ.

Metals. The analyses with a spark spectrograph were carried out by the Defence Standards Laboratory, Commonwealth Department of Supply, Melbourne. The dried crystals were placed in the hollowed bottom carbon electrode of the apparatus.

Iron was estimated according to Drabkin (1941) except that the final volume of the *o*-phenanthroline complex was brought to 5 ml.

Copper was determined by the following modification of the method of Sandell (1944). The samples (containing about 2 μg. of copper) were heated with 0·1 ml. of 17·5 N-H_2SO_4 until acid fumes appeared and were then digested for about 10 min. until colourless. The tubes were cooled, the contents were diluted with water and then neutralized with ammonia with methyl orange as an indicator. After addition of 0·5 ml. of N-H_2SO_4, the volume was brought to 5 ml. with water. About 3 ml. of dithizone (0·0012%) in carbon tetrachloride was added. The tubes were then shaken for 10 min. Extinction values were determined at 510 mμ, the absorption minimum of free dithizone.

Magnesium was estimated with Titan yellow according to Orange & Rhein (1951) except that the final volume was 2·5 ml.

Other experimental procedures used are described in previous papers (Appleby & Morton, 1954, 1959).

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RESULTS

Reaction of yeast lactic dehydrogenase with ferricyanide, cytochrome c and methylene blue

With 0·83 mM-potassium ferricyanide at pH 8·0, the rate of reduction appeared to be linear with time (Fig. 1, *A*). This suggests that the reaction is of zero order. However, a first-order reaction may be observed under other conditions.

With approx. 0·03 mM-cytochrome *c* (heart muscle) at pH 7·4, the rate of reaction decreased with time (Fig. 1, *B*). The plot of log (ferricytochrome *c* at zero time/ferricytochrome *c* remaining at time *T*) against *T* is linear (Fig. 1, *B*). Thus under these conditions the reduction of cytochrome *c* is a first-order reaction. The amount of cytochrome *c* reduced in the first minute was used as the estimate of the rate with this compound in routine assays.

In early studies with both crude and crystalline enzyme and with 0·02–0·03 mM-ferricytochrome *c*, the rate of reaction was apparently proportional to the amount of enzyme, up to that which produced a change of extinction at 549·5 mμ (see Appleby & Morton, 1959) of about 0·14 during the first minute. The specific activity so obtained with 0·02–0·03 mM-ferricytochrome *c* at 18–20° at pH 7·0 was 1955 units/mg. However, with 0·05 mM-cytochrome c (heart muscle) and with the assay system of Boeri, Cutolo, Luzzati & Tosi (1955), the specific activity of the crystalline enzyme at 20° was found to be 5870 units/mg. (i.e. approx. 2940 μmoles of lactate oxidized/hr./mg. of enzyme). This greater activity is due to the higher concentration of cytochrome *c* and to the protective effect of ethylenediaminetetra-acetate (EDTA) used in the assay system.

The rates of the reactions with ferricyanide, heart-muscle cytochrome *c* and methylene blue at

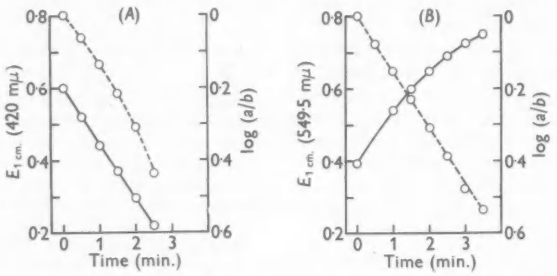

Fig. 1. Reduction of ferricyanide (*A*) and of heart-muscle ferricytochrome *c* (*B*), catalysed by yeast lactic dehydrogenase. ——, Extinction against time (*T*); - - - -, log (*a*/*b*) against time (*T*), where *a* is the concentration of oxidized acceptor at zero time, and *b* is the concentration remaining at time (*T*). (For conditions see text.)

Table 1. *Temperature coefficient and activation energy of the enzymically catalysed oxidation of lactate by ferricyanide, cytochrome c and methylene blue*

Twice-recrystallized enzyme was used. The rates of reduction of ferricyanide and of cytochrome *c* were measured spectrophotometrically with the constant-temperature cell holder of Dixon (1954); that of methylene blue was measured visually with Thunberg tubes held in a constant-temperature glass water bath. The methods are described by Appleby & Morton (1959). All assay mixtures contained mM-EDTA.

	Assay system					Temperature coefficient ($Q_{10°}$) and activation energy (μ)		
Hydrogen acceptor	Concn. of hydrogen acceptor (mM)	Buffer	pH	Temp.	Activity (units/ml.)	Temp. range	$Q_{10°}$	μ (kcal./mole)
Potassium ferricyanide	0·83	0·03 M-sodium pyrophosphate–HCl	8·0	20°	452	20–30°	1·5	7·2
				30	675			
Heart-muscle cytochrome *c*	0·05	0·05 M-sodium phosphate–HCl	7·0	20	342	20–30	1·5	7·6
				30	518			
Methylene blue	0·08	0·06 M-sodium acetate	5·2*	20	74	20–38	1·6	8·7
				38	170			

* This pH is not the optimum for this reaction: see Appleby & Morton, 1959.

two different temperatures are shown in Table 1, together with the temperature coefficients ($Q_{10°}$) for these reactions. The Arrhenius equation as given by Sizer (1943) was used with these measurements to calculate the activation energies shown in Table 1.

The optimum pH values for the reaction catalysed by a crude preparation of enzyme with ferricyanide, heart-muscle cytochrome *c* and methylene blue were 8·5, 6·0 and 5·2, respectively (Fig. 2). However, with the same acceptors, the pH optima were 8·0, 7·0 and 6·8 for crystalline enzyme (Fig. 3, see also, Appleby & Morton, 1959).

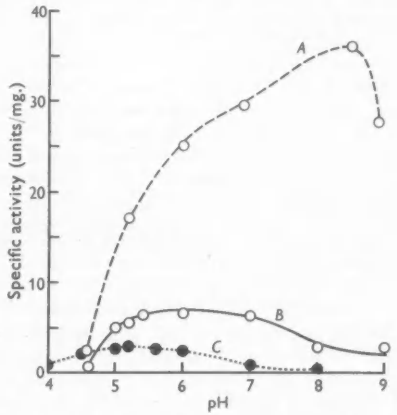

Fig. 2. Effect of pH on the rate of reduction of various hydrogen acceptors catalysed by a crude preparation of yeast lactic dehydrogenase. Results with 0·83 mM-potassium ferricyanide (curve *A*), heart-muscle ferricytochrome *c* (0·02 mM) (curve *B*) and 0·08 mM-methylene blue (curve *C*).

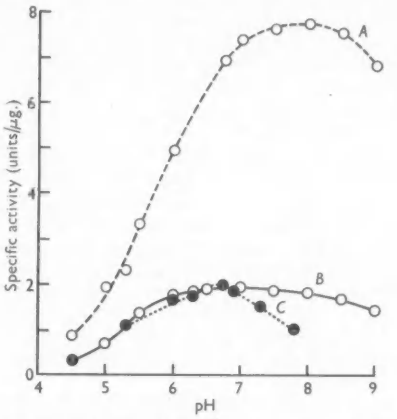

Fig. 3. Effect of pH on the rate of reduction of various hydrogen acceptors catalysed by crystalline yeast lactic dehydrogenase. Other details are as described for Fig. 2.

Reaction of yeast lactic dehydrogenase with oxygen

As observed by Bach *et al.* (1946), cytochrome b_2 is slowly auto-oxidizable. Freshly prepared crystals containing about 0·7% of sodium lactate were rapidly dissolved in a solution at 0° and at pH 7·4, previously equilibrated with air and containing 0·5 M-NaCl, 0·1 mM-EDTA and 0·01 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer. Spectroscopic observations showed that the cytochrome was completely oxidized in 2 min. Under these conditions, at pH 7·4 and at 0°, approx. 10 μmoles of lactate were oxidized/hr./mg. of enzyme (approx. 80 moles of lactate/hr./mole of enzyme). At pH 10·0 and at 15° auto-oxidation appeared to be much more rapid. At pH values less than 5·5, at which the enzyme is relatively slightly soluble, slow auto-oxidation as well as denaturation of the enzyme (see Appleby & Morton, 1959) was observed.

Substrate specificity of yeast lactic dehydrogenase

As shown in Table 2, of a range of salts of hydroxy acids, the crystalline enzyme reacted with only glycollate, lactate and α-hydroxy-*n*-butyrate. The highest activity was obtained with lactate itself.

The crystals had no diaphorase activity. No reduction of dye was observed in a system containing (in 3 ml.) 2 μmoles of reduced diphosphopyridine nucleotide, 0·15 μmole of 2:6-dichlorophenol-indophenol, 0·03 M-sodium pyrophosphate–acetic acid buffer, pH 8·5, and enzyme.

Table 2. *Substrate specificity of yeast lactic dehydrogenase*

A small pellet of freshly prepared, twice-crystallized enzyme was dissolved in a de-aerated solution at pH 6·8 and at 0° containing 0·5 M-NaCl and 0·1 mM-EDTA. The rate of reduction of potassium ferricyanide, in a solution containing 0·03 M-sodium pyrophosphate–HCl buffer, pH 8·0, mM-EDTA, 0·1 M-hydroxy acid (as the sodium salt) and either 0·01 or 0·1 ml. (as appropriate) of the solution of enzyme, was measured as previously described (Appleby & Morton, 1954, 1959). Activities are expressed as ferricyanide units/ml.

Substrate	Activity
None	0
Glycollate	144
DL-Lactate	2990
DL-α-Hydroxy-*n*-butyrate	1205
DL-β-Hydroxy-*n*-butyrate	0
α-Hydroxy-*iso*-butyrate	0

Absorption spectrum of crystalline cytochrome b_2

Fresh crystals or solutions of reduced yeast lactic dehydrogenase are pink, and change to red–brown on auto-oxidation. The absorption spectra of oxidized and lactate-reduced enzyme at pH 6·8 are shown in Fig. 4. The maxima at 556·5 and 528 mμ in the reduced enzyme correspond with the positions of the α- and β-bands of cytochrome b_2 as described by Bach *et al.* (1946). In addition, maxima occur at 424 and 330 mμ (γ- and δ-bands) and at 265 mμ. The values of ϵ at these maxima are given in Table 9.

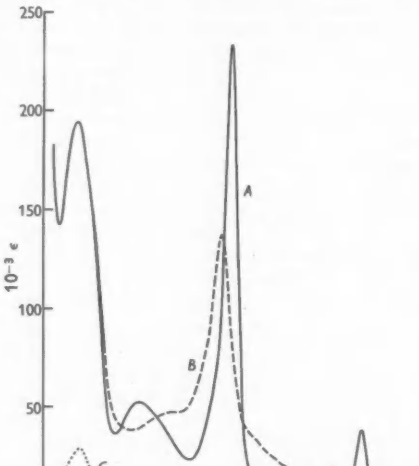

Fig. 4. Absorption spectra of riboflavin phosphate and of oxidized and lactate-reduced cytochrome b_2 (yeast lactic dehydrogenase). A Beckman spectrophotometer, model DU, was used and measurements were made in an atmosphere of nitrogen with Thunberg-type cuvettes with a light-path of 1 cm. (see Appleby & Morton, 1959). For curve *A* twice-crystallized enzyme was dissolved in M-sodium lactate, pH 6·8, and diluted in 0·1 M-sodium pyrophosphate, pH 6·8; the maxima are at 556·5, 528, 424, 330 and 265 mμ. For curve *B*, the preparation was dialysed against 0·1 M-sodium pyrophosphate–HCl buffer, pH 6·8, at 0° to remove lactate and then rapidly oxidized by aeration; the maxima are at 413, 359 and 265 mμ. In each case, the reference cuvette contained the appropriate buffer system, with or without lactate as necessary. For curve *C*, oxidized riboflavin phosphate, at a concentration equivalent to that of the flavin in the cytochrome b_2, was dissolved in 0·1 M-sodium pyrophosphate–HCl, pH 6·8; the maxima are at 445, 372 and 265 mμ. The extinction coefficient (ϵ) is given for a molar concentration of enzyme-bound haem. *A*, Lactate-reduced cytochrome b_2; *B*, oxidized cytochrome b_2; *C*, oxidized riboflavin phosphate.

The effect of pH on the absorption spectrum of the lactate-reduced cytochrome b_2 was investigated. At pH 5·0 there was no change from that found at pH 6·8, but at pH 10·0 an additional shoulder appeared near 450 mμ. This shoulder may be due to the presence of dissociated oxidized flavin.

It was observed spectroscopically that at the temperature of liquid air (see Keilin & Hartree, 1949) the visible absorption bands of the lactate-reduced cytochrome b_2 at pH 6·8 were considerably intensified. The α-band was shifted to 555·2 mμ (see Appleby & Morton, 1954), but no splitting of this band was detected, in contrast with the observation of other workers (Chance, Klingenberg & Boeri, 1956; Lindenmayer & Estabrook, 1958) with non-crystalline preparations of cytochrome b_2.

At pH 6·8 the oxidized enzyme has a parahaematin-type spectrum, with general absorption at 530–560 mμ, and with maxima at 413 and 359 mμ (Fig. 4). In the ultraviolet region, the absorption spectrum resembles that of the lactate-reduced enzyme.

Components of crystalline cytochrome b_2

Protohaem. Spectroscopic and spectrophotometric measurements of the reduced pyridine haemochromogen formed from crystalline lactic dehydrogenase showed a maximum (α-band) at 556 mμ, thus confirming the observations of Bach *et al.* (1942). The similar haemochromogens formed from crystalline protohaemin, and also from crystalline haemoglobin under identical conditions, showed maxima at 556·5 and 556 mμ, respectively.

For the reduced pyridine haemochromogen from cytochrome b_2, the maximum extinction of the α-band, and the ratio of this value to the minimum extinction near 540 mμ, was found to depend on the method of formation. When the enzyme was dissolved in the alkali before the addition of pyridine, the ratio of ($E_{max.}$ near 556 mμ)/($E_{min.}$ near 540 mμ) was 2·5 or less. When the pyridine was added to an aqueous suspension of freshly prepared or of freeze-dried crystals before the addition of NaOH and $Na_2S_2O_4$, the ratio was 3·45. For the reduced pyridine haemochromogen formed from crystalline protohaemin, Paul, Theorell & Åkeson (1953) obtained a ratio of 3·46, and ϵ for the α-band was $34{\cdot}2 \times 10^3$; in the present work, with crystalline protohaemin (8·2 % of iron), the values obtained were 3·43, and $33{\cdot}2 \times 10^3$, respectively.

Thus protohaem is the only haem present in crystalline yeast lactic dehydrogenase, which is a true cytochrome.

From the results obtained by adding NaOH and $Na_2S_2O_4$ to the enzyme in pyridine to form the reduced pyridine haemochromogen, and with the value for ϵ at 556 mμ of Paul *et al.* (1953), the

Table 3. *Haem, flavin and metal components of crystalline yeast lactic dehydrogenase*

Analyses were carried out on different batches of enzyme recrystallized at least twice. Methods used are described in the text. The proportion of component relative to haem is expressed as mole (or g.atom) of component/mole of haem.

Component	Assay procedure	Amount (%)	Proportion of component relative to haem	Wt. of enzyme/mole (or g.atom) of component estimated
Protohaem	Pyridine haemochromogen	0·77	(1·0)	80 000
Riboflavin phosphate	Fluorimetry (Bessey, Lowry & Love, 1949)	0·5	1·1	73 400
	Extinction at 445 mμ of			
	(1) heated extract		1·0	82 500
	(2) $HClO_4$ extract		1·0	80 500
Total iron	*o*-Phenanthroline complex (Drabkin, 1941)	0·091	1·3	61 400
Non-haem iron	By difference from haem		0·3	—
Copper	Sandell (1944)	0·016	0·2	—
Magnesium	Orange & Rhein (1951)	0·02	0·65	—

enzyme contains 0·77 % of haem. Hence the weight/mole of haem is approx. 80 000 g. (Table 3).

Riboflavin phosphate. When irradiated at about 360 mμ with a Hanovia lamp, solutions of freshly prepared enzyme did not fluoresce, whereas an intense yellow–green fluorescence, characteristic of free oxidized flavins, appeared after denaturation by either heat or acid. A solution of enzyme was extracted with phenol according to Crammer (1948), and the extract was examined by paper chromatography as described by Whitby (1950). Only riboflavin phosphate was detected. As determined by the fluorimetric method of Bessey, Lowry & Love (1949), only riboflavin phosphate (or a flavin of similar heat stability and solubility properties) was present; the amount (0·5 %) indicated a weight/mole of flavin of 73 400 g. (Table 3). Flavin was also estimated by measuring the total absorption at 445 mμ of the oxidized supernatant remaining after precipitation of the haem–protein both by heat denaturation and with perchloric acid. From these results (Table 3) a weight/mole of flavin of 82 500 g. and 80 500 g. respectively was obtained. Within the experimental error of the various determinations therefore, the protohaem and riboflavin phosphate are present in equimolecular proportions in the crystalline enzyme, which has a minimum molecular weight of about 80 000.

Iron and other metals. The results obtained with a spark spectrograph (see Methods section) showed that only iron, copper and magnesium were present in the crystalline enzyme in significant amounts (Table 4). These metals were therefore determined quantitatively by colorimetric methods as described in the Methods section.

The total iron content of an enzyme preparation crystallized twice was 0·091 %, corresponding to 1·3 g.atoms of iron/mole of enzyme haem. Thus the enzyme contains only 0·3 g.atom of non-haem iron/mole (Table 3).

Table 4. *Metal components of different preparations of crystalline yeast lactic dehydrogenase*

Twice-recrystallized enzyme was washed with 0·05 M-sodium lactate and the crystals were packed by centrifuging and then drained and freeze-dried. The material so obtained contained less than 1 % of lactate. It was placed directly in the hollow lower electrode of a spark spectrograph. The relative amount of each metal was estimated from photographs of the spark spectra.

Relative amount of metal	Metal components in Preparation 1* (enzyme of normal activity)	Metal components in Preparation 2* (enzyme of low activity)
Trace to strong trace	Si†	Cu
Trace	Cu, Mg	Fe, Na, Mg
Faint trace to trace	Fe	Zn
Faint trace	Ca	Ca
Very faint trace to faint trace	Al	—
Very faint trace	Zn, Pb	Ag
Very faint trace (doubtful)	Ag	—
Not detected	Mo, Co, Ni	Mo, Co, Ni, Al

* The activity of preparation 1 was approx. 6000 and that of preparation 2 was 2500 ferricyanide units/mg.

† A glass spatula was used to scrape this sample from the centrifuge tube.

A preparation with a specific activity of 6300 ferricyanide units/mg. contained 0·016 % of copper and 0·02 % of magnesium, corresponding to 0·2 and 0·65 g.atom of copper and magnesium respectively/mole of haem (Table 3).

Deoxyribose polynucleotide. The absorption at about 265 mμ (Fig. 3) in the crystalline enzyme is

due to riboflavin phosphate and a deoxyribose polynucleotide (Appleby & Morton 1954; Morton 1955; Appleby 1957). The isolation and identification of the polynucleotide (Appleby 1957) will be described in a subsequent paper.

Instability of yeast lactic dehydrogenase

The considerable instability of purified preparations of the enzyme was noted by Bach *et al.* (1946). It was observed during the development of methods for purification of the enzyme (see Appleby & Morton, 1959) that preparations of specific activity greater than 500 ferricyanide units/mg. rapidly lost activity, even when stored at 0°. There was less decline in activity if the enzyme was kept in the presence of excess of substrate, and further improvement was obtained by maintaining the enzyme under anaerobic conditions. These observations suggested that the loss of activity was due to hydrogen peroxide formed by auto-oxidation of the enzyme. It was found that hydrogen peroxide is indeed inhibitory, and that some protection was obtained with catalase (Table 5).

However, since anaerobic conditions reduced, but did not prevent, the loss of activity, it was apparent that inactivation was also caused by factors other than hydrogen peroxide. A solution of once-crystallized enzyme of specific activity 6620 ferricyanide units/mg. was dissolved in 0·03 M-sodium pyrophosphate containing 0·1 M-sodium lactate, pH 6·8, and dialysed anaerobically against 0·05 M-sodium lactate, pH 6·8, at 0°. Crystals appeared in 1 hr. but dialysis was continued for 5 days. The specific activity of the crystals so obtained was only 3920 ferricyanide units/mg. On irradiation, the mother liquor of these crystals and the diffusate both showed a strong yellow–green fluorescence, characteristic of free, oxidized flavin. Addition to the enzyme of substantial amounts of either cysteine-reduced riboflavin phosphate, or of cystein-reduced flavinadenine dinucleotide, failed to reactivate the preparation. The addition of 0·01 M-riboflavin phosphate to the 0·05 M-sodium lactate before dialysis (see Appleby & Morton, 1959) did not improve the yield or the specific activity of later preparations of crystals. Hence it appeared that the dissociation of riboflavin phosphate from the enzyme was irreversible.

There appeared to be a relationship between the loss of activity during storage, and the copper content of the crystalline enzyme. One preparation had a specific activity of 5500 ferricyanide units/mg. as determined immediately after dissolving the crystals in 0·5 M-sodium lactate at pH 6·8; the activity fell to 3800 units/mg. after holding aerobically for 1 hr. at 0°, and to 2500 units/mg. after further storage. At this stage the solution showed a strong flavin fluorescence when irradiated. As estimated with a spark spectrograph, this preparation of crystals had a high content of copper (Table 4, preparation 2). As shown in Table 6, the addition of mM-copper sulphate to a different preparation of crystalline enzyme caused almost complete inactivation after aerobic storage for 18 hr. at 0°. This inactivation was considerably reduced by addition of EDTA (Table 6), as was also the loss of activity caused by aerobic storage of enzyme in the absence of added Cu^{2+} ions (Table 7). The addition of EDTA (about 0·1 mM) also considerably reduced the rate of inactivation of enzyme stored under anaerobic conditions.

The following observations support the finding that the metal-catalysed auto-oxidation of cytochrome b_2 is accompanied by dissociation of riboflavin phosphate. A pellet of lactate-reduced crystals was freshly prepared in the absence of EDTA. The pellet was pink and did not fluoresce on irradiation. After exposure to air at 0° the colour changed to orange, as oxygen diffused through the pellet. The well-defined front of the orange oxidized cytochrome corresponded exactly with the zone of yellow–green fluorescence as seen on irradiation of the crystals. However, a similar pellet of crystals prepared in the presence of 0·1 mM-EDTA showed only faint fluorescence in the zone of oxidized cytochrome.

Table 5. *Effects of catalase and of added hydrogen peroxide on the activity of yeast lactic dehydrogenase*

A solution of once-crystallized enzyme (approx. 40 μg./ml.) in M-sodium lactate, pH 6·8, was used. About 100 μg. of crystalline liver catalase (Armour Laboratories, U.S.A.)/ml. was added to a portion of the enzyme solution. The solutions were stored aerobically. The rate of reduction of potassium ferricyanide in 0·03 M-sodium pyrophosphate–acetic acid buffer, pH 8·0, was measured as previously described (Appleby & Morton, 1954, 1959). Activities are expressed as ferricyanide units/ml.

Stage	Storage and treatment conditions	Enzymic activity: With catalase	Enzymic activity: Without catalase
1	Fresh preparation	248	248
2	Held for 4 days at 0°	230	94
3	Approx. 0·2 mM-H_2O_2 was added to material treated as described above, then held for 10 min. at 0°	198	78
4	H_2O_2 increased to mM concentration; enzyme was then held for 10 min. at 0°	184	29·5

Prosthetic groups of yeast lactic dehydrogenase

Riboflavin phosphate. The observations cited above indicate that protein-bound riboflavin phosphate is essential for enzymic activity and

Table 6. *Inactivation of yeast lactic dehydrogenase by Cu^{2+} ions and protection by ethylenediaminetetra-acetate*

A solution of enzyme (crystallized once) in 0·5 M-sodium lactate, pH 6·8, was used. All tubes, except the controls, were aerated. In each experiment, oxygen was displaced from the control tube with nitrogen. The rate of reduction of ferricyanide was estimated (Appleby & Morton, 1954, 1959) with 0·03 M-sodium pyrophosphate–HCl, pH 8·0, and at 18°. Activities are expressed as ferricyanide units/ml.

In Expt. 1, copper sulphate was added, and in Expt. 2, copper sulphate and EDTA were added, to give the concentration shown. All tubes were stored for 18 hr. at 0°.

Expt. 1			Expt. 2			
	Enzymic activity				Enzymic activity	
Concn. of $CuSO_4$ (μM)	Anaerobic storage	Aerobic storage	Concn. of $CuSO_4$ (μM)	Concn. of EDTA (μM)	Anaerobic storage	Aerobic storage
0	224	186	0	0	179	119
0·1	—	167	10	0	—	10
10	—	57	10	1	—	10
1000	—	2	10	10	—	97
			10	100	—	195

Table 7. *Protection of yeast lactic dehydrogenase by ethylenediaminetetra-acetate*

A preparation of once-crystallized enzyme was dissolved in 0·5 M-sodium lactate, pH 6·6, and EDTA was added to give the concentrations shown below. Activity with ferricyanide (Appleby & Morton, 1954, 1959) was determined with 0·03 M-sodium pyrophosphate–HCl buffer, pH 8·0, at 18°, immediately and after holding the preparation aerobically for 18 hr. at 0°. Activities are expressed as ferricyanide units/ml.

Concn. of EDTA (μM)	Enzymic activity	
	At commencement	After storage (18 hr.)
0	366	80
0·1	370	258
1	—	262
10	383	320
100	460	465

suggest that riboflavin phosphate acts as a hydrogen acceptor in the oxidation of lactate.

With oxidized lactic dehydrogenase, the intense Soret band due to the haem group (Fig. 4, curve *B*) prevents observation of the expected absorption bands at about 375 and 450 mμ due to oxidized riboflavin phosphate. For the same reason, the difference spectrum (oxidized minus reduced enzyme) is not that of a typical flavoprotein. However, for the lactate-reduced enzyme, ϵ at 450 mμ is only $6{\cdot}2 \times 10^3$ (Fig. 4, curve *A*). If the riboflavin phosphate was not reduced by lactate, the minimum value of ϵ expected at this wavelength, due to oxidized riboflavin phosphate, would be $11{\cdot}2 \times 10^3$ (Fig. 4, curve *C*). Hence the flavin group must be at least partly reduced by lactate. This is supported by the following observations. A freshly prepared pellet of crystalline enzyme was dissolved in 0·5 M-NaCl containing 0·1 mM-EDTA, pH 6·8, at 0°. Measurements of the visible absorption bands were made with a Hartridge reversion spectroscope as the solution was gently shaken in the air. The bands of the reduced enzyme at about 556·5 and 527 mμ slowly faded and were replaced by broad but weak absorption bands at approx. 567 and 533 mμ. These bands disappeared completely on further oxidation. The bands at 556·5 and 527 mμ reappeared immediately on the addition of a small amount of lactate. The bands at 567 and 533 mμ are not seen in the fully oxidized enzyme, which has a parahaematin-like spectrum (Fig. 4, curve *B*), and they are observed only momentarily as the fully reduced enzyme is oxidized. Hence they are the absorption bands of the enzyme at an intermediate state of oxidation. As far as is known (see Morton, 1958), no other cytochromes show similar changes during oxidation of the reduced compound. However, Beinert (1957) reported that some flavoprotein enzymes have a single absorption band near 560 mμ which he ascribed to the semiquinoid form of the flavin prosthetic group. Although Beinert (1957) did not report any band near 533 mμ, it seems probable that the bands at 567 and 533 mμ indicate the formation of a semiquinone of riboflavin phosphate during oxidation of lactic dehydrogenase and hence the participation of flavin in the enzymic reaction.

Storage of oxidized enzyme at 0° in the absence of EDTA caused rapid loss of activity, as measured by reduction of ferricyanide, and, correspondingly, a decline in the amount of haem capable of reduction by lactate on incubation for about 10 min. at 0° (Table 8). However, there was little change in the amount of haem reducible with $Na_2S_2O_4$ (Table 8). The rate of reduction of ferricyanide declined about twice as rapidly as did the amount of haem reducible by lactate in 10 min. at 0°. The sample stored for 18 hr. (Table 8) showed a strong greenish fluorescence on irradiation, indicating

Table 8. *Effects of aerobic storage on the reduction of the haem group of yeast lactic dehydrogenase by lactate and by dithionite*

Freshly recrystallized enzyme was suspended in water at 0° and held aerobically at 0°. At the times indicated, portions (0·1 ml.) were diluted 50-fold in 0·5 M-sodium lactate containing 0·1 mM-EDTA, pH 6·8, at 0°. The rate of reduction of ferricyanide (in 0·03 M-sodium pyrophosphate–HCl buffer, pH 8·0, containing 0·1 mM-EDTA) was measured immediately. Measurements were made of the position and height of the Soret band, and of the extinction at 424 mμ, at approx. 10 min. after preparation of the dilution and after the further addition of about 2 mg. of $Na_2S_2O_4$ to the cuvette (capacity 3 ml.) containing the diluted enzyme. It was assumed that the cytochrome was fully reduced by $Na_2S_2O_4$ and the proportion of the haem reduced by lactate was calculated by assuming the ratio of extinction values at 424 mμ for oxidized enzyme/reduced enzyme to be 0·354 (see Fig. 4). The extinction values are given for the undiluted preparation.

Activities are expressed as ferricyanide units/ml. and wavelengths are in mμ.

			Extinction values for Soret band					
			Reduced with lactate			Reduced with $Na_2S_2O_4$		
				E				
Storage time at 0°	Activity (units/ml.)	Relative activity after storage (%)	$\lambda_{max.}$* (mμ)	At $\lambda_{max.}$	At 424 mμ	$\lambda_{max.}$* (mμ)	$E_{424\ m\mu}$	Proportion of haem group reduced by lactate (%)
0	23800	(100)	424	11·5	11·5	424	11·0	(105)
10 min.	13210	55	423·5	10·1	10·1	424	11·0	87
18 hr.	5050	21	419	7·4	6·5	424	10·7	43

* $\lambda_{max.}$ indicates the wavelength at which the maximum extinction of the Soret band was obtained.

some dissociation of the flavin group. These observations support the suggestion (Appleby & Morton, 1954) that riboflavin phosphate, and not protohaem, acts as the primary acceptor of hydrogen atoms from lactate.

Protohaem. It was observed spectroscopically that the addition of ferricyanide to a dilute solution of cytochrome b_2 in excess of lactate caused the immediate disappearance of the α- and β-bands of the reduced enzyme. When all the ferricyanide was reduced, these bands reappeared. They disappeared finally when the lactate was completely oxidized. Hence the haem group undergoes reversible oxidation and reduction during the oxidation of lactate.

Numerous attempts to separate lactic dehydrogenase activity from cytochrome b_2 were unsuccessful (Appleby & Morton, 1954, 1959).

DISCUSSION

The crystallization of cytochrome b_2 and identification of riboflavin phosphate and protohaem as the prosthetic groups was first reported by Appleby & Morton (1954). The evidence described here and earlier (Appleby & Morton, 1954, 1959; Appleby, 1957) establishes that cytochrome b_2 is identical with yeast lactic dehydrogenase and that the enzyme contains flavin and haem in equimolecular proportions. This finding has since been confirmed by other workers (Boeri *et al.* 1955; Boeri & Tosi, 1956; Nygaard, 1958) with non-crystalline enzyme preparations.

Yamashita *et al.* (1957) have described a crystalline preparation of a yeast haemoprotein which these workers called 'cytochrome b_2' but which had no lactic dehydrogenase activity. Bach *et al.* (1946) first observed that preparations in which the haem group was reducible by dithionite, but not by lactate, could be obtained by denaturation of yeast lactic dehydrogenase. Loss of enzymic activity, accompanied by the appearance of fluorescence due to free flavin, was frequently observed by us during investigation of chromatographic methods for purification of the enzyme (see also, Nygaard, 1958). Moreover, when we subjected crystalline lactic dehydrogenase to chromatographic procedures rather similar to those of Yamashita *et al.* (1957), enzymic activity was lost and free flavin (not flavoprotein) was detected in the eluates. In addition to protein denaturation, metal-catalysed oxidation causes rapid dissociation of the flavin group of the enzyme accompanied by loss of enzymic activity (Tables 6–8); however, the haem group remains reducible by dithionite (Table 8). In view of the use of autolysis for 1 week at pH 8·5 during purification (Yamashita *et al.* 1957), it is not surprising that the haemoprotein obtained by these workers does not contain riboflavin phosphate (Yamanaka, Horio & Okunuki, 1958). The material isolated by Yamashita *et al.* (1957) thus appears to be a product derived from the native flavohaemoprotein, cytochrome b_2. Our work gives no support for the suggestion of Yamanaka *et al.* (1958) that the crystalline lactic dehydrogenase of Appleby & Morton (1954, 1959) is a complex of a haemoprotein ('cytochrome b_2') and a flavin enzyme.

Table 9 compares the properties of the crystalline lactic dehydrogenase of Appleby & Morton (1954,

Table 9. *Comparative properties of several preparations of yeast lactic dehydrogenase.*

In each case the values refer to the preparation of maximum purity and to enzymic activities determined under optimum conditions as defined by the various authors. Wavelengths are given in mμ: the extinction coefficient (ε) in each case is given for a molar concentration of enzyme-bound haem.

Property	Values according to authors indicated: Appleby & Morton (1954, 1959 and this paper)	Boeri, Cutolo, Luzzati & Tosi (1955) and Boeri & Tosi (1956)	Bach, Dixon & Zerfas (1946)
Chemical and physical properties:			
Weight of enzyme (g.)/mole of haem	80 000	230 000	850 000
No. of prosthetic groups/minimum weight of enzyme:			
Protohaem	1	1	1
Riboflavin phosphate	1	1	—
Non-haem iron	0*	8	—
Deoxyribose nucleotide	1†5	—	—
Principal absorption bands of reduced enzyme:			
α-Band λ	556·5	557	556·5
α-Band $10^{-3}\,\epsilon$	38·8	36	32·6
β-Band λ	528	528	5[illegible]0
β-Band $10^{-3}\,\epsilon$	18·6	25·4	22·2
γ-Band λ	424	423	—
γ-Band $10^{-3}\,\epsilon$	232	224	—
δ-Band λ	330	—	—
δ-Band $10^{-3}\,\epsilon$	52·2	—	—
Ultraviolet-band λ	265	—	—
Ultraviolet-band $10^{-3}\,\epsilon$	199	—	—
Enzymic activities:			
With heart-muscle cytochrome *c* at 20°			
pH optimum	7·0	7·0–8·0	—
μmoles reduced/hr./mg. of enzyme	5 870	3 660	—
moles reduced/min./mole of enzyme-bound haem	7 850	14 000‡	—
With potassium ferricyanide at 20°			
pH optimum	8·0	7·0–8·0	—
μmoles reduced/hr./mg. of enzyme	7 750	3 660	—
moles reduced/min./mole of enzyme	10 350	14 000‡	—
With methylene blue at 38°			
pH optimum	6·8	5·8	5·3
μmoles reduced/hr./mg. of enzyme	2 000	580	—
moles reduced/min./mole of enzyme-bound haem	2 700	2 200	—
Q_{MB}§	56 800	—	3000

* Actual values of 0·1 (unpublished studies of J. McD. Armstrong & R. K. Morton) to 0·3 g.atoms of non-haem iron/mole of haem (Table 3) are regarded as indicating slight impurity only.

† Appleby (1957).

‡ These high values reflect the low haem iron found by Boeri *et al.* (1955).

§ As defined by Bach *et al.* (1946).

1959) with those of the non-crystalline enzyme preparations of other workers (Bach *et al.* 1946; Boeri *et al.* 1955). Electrophoretic and ultracentrifuge studies, and amino acid analyses, which will be described in subsequent papers, provide further evidence of the homogeneity of the crystalline enzyme of Appleby & Morton (1954, 1959). The weight of the crystalline enzyme/mole of haem or of riboflavin phosphate (about 80 000 g.) is rather similar to the weight/mole of flavin of a number of the purified flavoprotein enzymes, such as 'old yellow enzyme' of yeast (52 000 g.; Theorell, 1935; Theorell & Åkeson, 1956), reduced triphosphopyridine nucleotide–cytochrome *c* reductase of yeast (75 000 g.; Haas, Horecker & Hogness, 1940) and D-amino acid oxidase of kidney (69 000 g.; Blanchard, Green, Nocito & Ratner, 1944). On the basis of the reaction of yeast lactic dehydrogenase with cytochrome *c*, Green & Beinert (1955) predicted that the enzyme contains a metal chelated by the flavin group. However, the analyses in Tables 3, 4 and 9 show that there is insufficient non-haem iron and copper, and insufficient of any other likely metal, for a function in mediating

electron transfer as proposed by Green & Beinert (1955). Boeri & Tosi (1956) propose that the 8 g.-atoms of non-haem iron in the lactic dehydrogenase of Boeri *et al.* (1955) (see Table 9) have an essential function in mediating reactions with cytochrome *c* (cf. Mahler & Green, 1954; Mahler & Elowe, 1954). However, the results presented here and in summary earlier (Morton, 1955) strongly suggest that the non-haem iron found by Boeri & Tosi (1956) is an impurity of the preparation. Moreover, we obtained no stimulation of the activity with cytochrome *c* by addition of ferrous sulphate to the crystalline enzyme (Appleby, 1957). It is considered that the magnesium (approx. 1 g.atom/mole of haem; Table 4) is probably present in non-specific association with the polynucleotide component of the cytochrome (see Table 9).

The extinction values for the principal absorption bands of crystalline cytochrome b_2 (Fig. 4 and Table 9) are greater than those of any similar cytochromes as yet purified (see Morton, 1958, Table 5). By spectrophotometric titration with lactate of a non-crystalline preparation of cytochrome b_2 (Boeri *et al.* 1955), ϵ for the reduced minus oxidized preparation at 557 mμ was estimated by Chance *et al.* (1956) as 21×10^3; the value at pH 6·5–6·8 for the crystalline enzyme is $23{\cdot}9 \times 10^3$ (see Fig. 4). The quotient $E_{556{\cdot}5\ m\mu}/E_{550\ m\mu}$ for crystalline cytochrome b_2 of Appleby & Morton is 1·8–1·9; for the preparation of Bach *et al.* (1946) it is 1·6; and for that of Boeri *et al.* (1955) it is 1·4. As higher values generally reflect a greater purity, it would appear that the preparation of Boeri *et al.* (1955) may contain some cytochrome *c* or cytochrome c_1 or similar type of impurity. Table 9 shows that the specific activity (μmoles of acceptor reduced/hr./mg. of enzyme) of the preparation of Appleby & Morton is very much higher than that of Boeri *et al.* (1955) with all acceptors. However, the activity as moles of cytochrome *c* reduced/min./mole of haem is greater for the preparation of Boeri *et al.* (1955). This may indicate difficulties in estimation of protohaem or of enzymic activity, or the presence in the cruder preparation of factors such as yeast cytochrome *c* or c_1, which stimulate the activity with heart-muscle cytochrome *c*.

As shown by Table 2, glycollate and α-hydroxybutyrate, as well as lactate, are substrates of the enzyme. It has also been shown by J. McD. Armstrong & R. K. Morton (unpublished work) that certain other hydroxy acids, including L-malic acid and DL-glyceric acid, and also reduced di- and triphosphopyridine nucleotide, are not substrates of the crystalline enzyme. Contrary findings by other workers (Yamashita *et al.* 1957; Yamanaka *et al.* 1958; Yamashita, Horio & Okunuki, 1958) must be attributed to contaminating enzymes in the preparations used. Dickens & Williams (1956) recognized that the oxidation of L-glyceric acid by a preparation of yeast lactic dehydrogenase according to Boeri *et al.* (1955) was possibly due to an enzymic impurity.

The enzyme is specific for the L-isomer of lactate (Mahler & Huennekens, 1953; Boeri *et al.* 1955). The oxidation of L-lactate is not inhibited by D-lactate (Boeri *et al.* 1955) so that DL-lactate is suitable as a substrate for enzymic studies.

Several of the findings described here relate to the function of riboflavin phosphate in cytochrome b_2 (Appleby & Morton, 1954). Inactivation of the enzyme accompanied by dissociation of the flavin prosthetic group is caused by oxidation by hydrogen peroxide or by Cu^{2+} ions under aerobic conditions (Tables 5, 7 and 8). Whereas the enzyme is strongly inhibited by *p*-chloromercuribenzoate (0·1–10·0 mM) (Appleby & Morton, 1954; Boeri *et al.* 1955), monoiodoacetate (10 mM) under similar conditions had little effect on the activity (Boeri *et al.* 1955). This suggests that the thiol groups of the enzyme are masked or bound (see Barron, 1951). These several observations are in accord with the hypothesis that the flavin is linked to the apoenzyme by a bond between the imino group of the *iso*alloxazine of the riboflavin phosphate (see Theorell, 1955) and the thiol group of a cysteine residue of the apoenzyme. Oxidation of the enzyme and partial dissociation of the oxidized riboflavin phosphate (see Vestling, 1955) would expose the –SH groups. Metal-catalysed oxidation of the –SH groups would thus lead to irreversible displacement of the riboflavin phosphate, as observed. The protective effect of ethylenediaminetetra-acetate (Table 6), which was also reported by Boeri *et al.* (1955), is attributed to the prevention of metal-catalysed oxidation of the thiol groups.

Marcus & Vennesland (1958) have shown that the reaction catalysed by yeast lactic dehydrogenase does not involve hydrogen atoms attached to the β-carbon atom of lactate. Presumably the lactate molecule is orientated at the enzyme surface in such a way as to permit the transfer of a hydrogen atom from the α-carbon atom to oxidized riboflavin phosphate. The flavin semiquinone so formed would no doubt have a transient existence. However, the probable formation of such an intermediate is supported by the direct spectroscopic observation of an unstable oxidation product of the enzyme, and by the recent demonstration of free radicals formed during oxidations catalysed by several different flavoprotein enzymes (Commoner, Lippincott & Passonneau, 1958).

In fully active preparations of the enzyme both the flavin and haem group appear to be involved in the reduction of ferricyanide, cytochrome *c* and methylene blue. Preliminary determinations of the activation energies (Table 1) suggest that the same

rate-limiting step is involved in the reactions with each of these acceptors. Furthermore, spectrophotometric titration of cytochrome b_2 with lactate (Chance *et al.* 1956) indicates that both the flavin and haem group are reduced simultaneously. However, under certain conditions of inactivation, the rate of reduction of ferricyanide declines more rapidly than does the rate of reduction of the haem group of the enzyme (Table 8); the haem group of inactive cytochrome b_2 may be slowly reduced by active enzyme (Appleby & Morton, 1954; Yamashita *et al.* 1957, 1958). The claim that non-haem iron (Boeri & Tosi, 1956) or other metals (Green & Beinert, 1955) have an essential function in mediating electron transport between the flavin and haem prosthetic group is not substantiated by the results reported here.

Of the other known cytochrome *c*-linked dehydrogenases, succinic dehydrogenase of heart muscle and aldehyde dehydrogenase of pig liver merit comparison with yeast lactic dehydrogenase. Succinic dehydrogenase is associated with cytochrome *b* in heart muscle, but was separated from this by treatment of the lipoprotein complex with butan-1-ol (Morton, 1950, 1955) and shown to be a flavoprotein (Morton, 1955; Kearney & Singer, 1955; Wang, Tsou & Wang, 1956). The purified succinic dehydrogenase contains 4 g.atoms of non-haem iron/mole of enzyme, but this iron does not appear to be involved in electron transfer (Massey, 1958).

Mahler (1956) has stated that liver aldehyde dehydrogenase contains flavin, metal (molybdenum) and haem groups, and thus resembles yeast lactic dehydrogenase. However, the haem content of the aldehyde dehydrogenase is extremely variable in different preparations; in the reduced state, this enzyme shows only very weak visible absorption bands; the difference spectrum is that of a typical flavoprotein (Mahler, Mackler, Green & Bock, 1954). There is as yet no evidence that the haem group is reversibly oxidized and reduced during catalysis by this enzyme.

Yeast lactic dehydrogenase, being a true cytochrome and having both flavin and haem prosthetic groups, thus differs from both succinic and aldehyde dehydrogenases, and from any other dehydrogenase as yet described.

SUMMARY

1. Chemical and enzymic studies with crystalline yeast lactic dehydrogenase are described. The absorption spectrum of the lactate-reduced enzyme shows maxima at 556·5, 528, 424, 330 and 265 mμ; ε at 556·5 mμ is $38{\cdot}8 \times 10^3$. There is 1 mole each of protohaem and of riboflavin phosphate present as prosthetic groups/80 000 g. of enzyme. No significant amount of non-haem iron, copper, cobalt, molybdenum or other related metal is present. There is 1 g.atom of magnesium/80 000 g. of enzyme; this is probably associated with the deoxyribopolynucleotide component of the crystalline material.

2. Oxidation by hydrogen peroxide, and by added Cu^{2+} ions under aerobic conditions, causes dissociation of the flavin prosthetic group and loss of dehydrogenase activity. Ethylenediaminetetraacetate protects against metal-catalysed inactivation. The mechanism of the inactivation process is discussed.

3. The term cytochrome b_2 applies to the native, enzymically active flavohaemoprotein. A haemoprotein product containing no riboflavin phosphate and devoid of enzymic activity may be obtained from cytochrome b_2.

4. The enzyme catalyses the oxidation of glycollate, L-lactate and α-hydroxybutyrate only. Lactate is oxidized most rapidly.

5. The activation energies for reduction of heart-muscle cytochrome *c*, potassium ferricyanide and methylene blue under specific conditions are 7·2, 7·6 and 8·7 kcal./mole, respectively. Under specified conditions, reduction of heart-muscle cytochrome *c* proceeds by a first-order reaction, and of ferricyanide by a zero-order reaction.

6. The enzymic and chemical properties of the crystalline cytochrome b_2 are compared with those of non-crystalline preparations obtained by other workers.

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The Composition of Plum Gums

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Gummosis, or the formation of gums, is a common phenomenon in the plant kingdom, and in temperate climates fruit trees of the Rosaceae family, e.g. peach, plum, almond, etc., are particularly susceptible. These gums may appear as exudates on the epidermal surfaces of the various plant organs or as internal pockets ('stone gum') within the tissues. They are composed predominantly of carbohydrate in the form of inorganic salts of complex acidic polysaccharides.

The biosynthetic mechanisms involved in the formation of gum polysaccharides are not understood. It has been variously suggested that gums are the products of micro-organisms (Grieg-Smith, 1904; Butler & Jones, 1949) and that they are produced from pectin (Higgins, 1919) and from hemicellulose (Sands & Gary, 1933). Recent histochemical studies by Ceruti & Scurti (1954) have shown that the precursor of cherry gum (*Prunus avium* L.) is probably a galactan. There seems little doubt that the gums originating from *Prunus* species are metabolic products of the plant normally found in the tissues but produced in excess when the plant is stimulated by mechanical or microbiological injury (Hirst, 1949) or by some physiological disorder. The apparent function of gum formation is to seal off surface wounds and to prevent the spread of invading micro-organisms.

This paper describes some studies on the composition of plum gums in relation to their possible function in the plant. A preliminary account of this work has been given by Pridham (1954).

MATERIALS AND METHODS

General methods. M.p. values are uncorrected. Optical rotations are given as equilibrium values in aqueous solution at 25°. Evaporations were carried out under reduced pressure. A Unicam SP. 500 spectrophotometer (1 cm. cell) was used for all u.v. measurements.

Purification of the gum polysaccharides. The gums, which were collected from trees (var. Victoria) were vigorously shaken with water at room temperature, the solutions were clarified in the centrifuge and the polysaccharides precipitated from the supernatant liquors by addition of excess of ethanol. After isolation by centrifuging, the white precipitates were washed with absolute ethanol followed by ether, and dried at 60° under reduced pressure.

Hydrolysis of the gum polysaccharides. (i) Qualitative. The gums were hydrolysed in N-H_2SO_4 in sealed glass tubes for 16 hr. After neutralization ($BaCO_3$) and concentration, the residues were examined on paper chromatograms. Barium ions were removed from the hydrolysates by treatment with Amberlite IR-120 (H) resin and, after concentration, the residues were again examined on paper chromatograms for glucurono-γ-lactone, which appeared as a characteristic red spot [$R_{\text{galactose}}$ 4·8 in solvent (1), see below] with the *p*-anisidine hydrochloride reagent. Further characterization of the monosaccharide constituents of the fruit-surface gum was carried out as follows. The gum (5 g.) was hydrolysed in N-H_2SO_4 (160 ml.) at 95–100° for 21 hr. After neutralization ($BaCO_3$) the solution was evaporated to a thick syrup which was dried by repeated evaporation in the presence of methanol. The resulting material was then extracted with boiling methanol (100 ml.; three portions) to separate the neutral monosaccharides (*A*) from the insoluble barium salts (*B*). Evaporation of the filtered methanol extracts gave a mixture of sugars (*A*; 2·54 g.) which was fractionated by chromatography on a column of cellulose with aq. butan-1-ol (half-saturated with water) as the mobile phase. The barium salts (*B*, 4·46 g.) were dissolved in water and then passed through a column of Amberlite IR-120 (H) resin, and the acidic effluent was concentrated to a small volume. After examination on paper chromatograms this solution was passed through a charcoal–Celite column, which was washed with water to elute D-glucurono-γ-lactone. Further elution of the column with aq. 50% ethanol afforded a mixture of oligosaccharides (*C*, 139 mg.). A portion (39 mg.) of this mixture (*C*) was hydrolysed (2 N-H_2SO_4, 100°, 16 hr.) and the neutralized ($BaCO_3$) hydrolysate examined on paper chromatograms. The remainder of this mixture (*C*, 100 mg.) was heated under reflux with methanolic 4% hydrogen chloride until the solution was non-reducing to Fehling's solution. The neutralized (Ag_2CO_3) solution was concentrated, the resulting syrup was dissolved in tetrahydrofuran and treated with lithium aluminium hydride (Lythgoe & Trippett, 1950). Excess of hydride was decomposed with ethyl acetate, the solution was poured into ice–water and then filtered. Concentration gave a mixture of methyl glycosides which were hydrolysed (N-H_2SO_4, 100°, 16 hr.) and then examined on paper chromatograms.

(ii) Quantitative. (*a*) The fruit-surface and bark gums were hydrolysed in N-H_2SO_4 by heating in an autoclave (15 lb./in.², 120°) for exactly 2 hr. The hydrolysates were neutralized with Amberlite IR-4B (OH) and the sugars determined by the benzidine method (Jones & Pridham, 1954). (*b*) The analyses were repeated, as described above, but the period of hydrolysis was reduced to 1 hr. with N-H_2SO_4 at 15 lb./in.² pressure.

Paper chromatography. All separations were carried out on Whatman no. 1 paper by the descending method, unless otherwise stated, with one of the following solvent systems: (1) ethyl acetate–acetic acid–water (9:2:2, by vol.); (2) ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.); (3) butan-1-ol–pyridine–water (10:3:3, by vol.); (4) butan-1-ol–ethanol–water (40:11:19, by vol.); (5) phenol–water (bottom layer); (6) butan-1-ol–acetic acid–water (2:1:1, by vol.); (7) propan-1-ol–water (4:1, v/v); (8) light petroleum (b.p. 100–110°) saturated with water. Solvents (5) and (6) were used for the examination of amino acids which were detected by the use of ninhydrin.

Reducing sugars were located on the paper chromatograms with the following spray reagents: (*a*) ammoniacal $AgNO_3$ (Partridge, 1948); (*b*) *p*-anisidine hydrochloride; (*c*) resorcinol–HCl (Hough, Jones & Wadman, 1950).

Phenols and their derivatives were separated by using solvents (4) and (6) and they were located on the paper chromatograms with diazotized *p*-nitroaniline–Na_2CO_3 (Swain, 1953) and diazotized sulphanilic acid (Evans, Parr & Evans, 1949) spray reagents. In some cases the spots were also detected by their fluorescence under ultraviolet light with or without exposure to NH_3.

Phosphates were examined on Whatman no. 542 acid-washed paper, irrigated with solvent (2) and subsequently detected by the procedure of Hanes & Isherwood (1949).

Alkaline nitrobenzene oxidation products were detected on paper chromatograms with 2:4-dinitrophenylhydrazine solution (0·3% in 2 N-HCl, Stone & Blundell, 1951).

The inorganic cations present in fruit-surface gum and stone gum of plums were studied by the qualitative paper chromatographic procedures described by Pollard & McOmie (1953).

Hexuronic acid anhydride content. The method of Johannson, Lindberg & Theander (1954) was used, and each sample of gum (approx. 0·1 g.) analysed in duplicate. The results were corrected for ash and water content of the gums.

Periodate oxidations. A known weight of the gum was dissolved in water by vigorous agitation at room temperature; 0·3 M-sodium metaperiodate solution (20 ml.) was added and the volume made to 100 ml. with water. A control solution containing 0·3 M-sodium metaperiodate solution (20 ml.) and water (80 ml.) was prepared at the same time. The oxidation was carried out in the dark and at room temperature. At varying intervals of time, two samples (5 ml. each) were withdrawn from each flask. To one sample ethylene glycol (2 ml.) was added; it was then diluted to about 12 ml. with water and the acidity titrated potentiometrically with 0·01 N-NaOH (Anderson, Greenwood & Hirst, 1955). The other sample was added to a mixture of 20% KI solution (5 ml.) and 2 N-H_2SO_4 (5 ml.) and the periodate content was determined by titration of the liberated iodine with 0·1 N-sodium thiosulphate (Schwartz, 1954). Oxidations were carried out on crude and purified gums.

Electrophoresis. The gum was dissolved in aqueous buffer solution by vigorous agitation at room temperature. After centrifuging, the solution was dialysed against buffer until the pH and conductivity of both buffered gum solution and the dialysate were approximately the same. Electrophoresis was carried out at 4° in a Hilger electrophoresis apparatus at a potential of 280–380 v and a current of 12–18 ma.

Mono- and oligo-saccharides. Crude gums were finely powdered and extracted by shaking with methanol at room temperature for 20 hr. The filtered extracts were concentrated and examined on paper chromatograms. In one instance extraction was effected with methanol containing about 1% of aq. NH_3 soln.

Lignin. Crude gum was heated with 2 N-H_2SO_4 at 100° for 16 hr., care being taken to see that the samples did not contain any plant tissue. The residue was then filtered off and subjected to alkaline nitrobenzene oxidation (Freudenberg, Lautsch & Engler, 1940). The resulting products were examined on paper with solvent (8) and 2:4-dinitrophenylhydrazine as spray reagent.

Phenolic compounds. The gum was agitated with an excess of water at room temperature, the solution was clarified by centrifuging, acidified with acetic acid and then extracted continuously with ether for 10 hr. After concentration, the ethereal extract was examined on paper chromatograms. Further extraction of the aqueous gum solution was then carried out with ethyl acetate: this extract and the residual aqueous solution were concentrated and the syrups examined on paper chromatograms.

Amino acids and protein. Several specimens of gum were analysed for nitrogen. Gums were examined by paper chromatography, before and after partial hydrolysis, for ninhydrin-positive compounds. Partial hydrolysis was effected by leaving a 1% solution of gum in 3 N-HCl at room temperature for 3 weeks. The acid was neutralized (Ag_2CO_3) and the solution then treated with H_2S and concentrated.

Peroxidase. Benzidine (Madeling, 1911) and pyrogallol (Willstätter & Stoll, 1918), both in the presence of traces of H_2O_2, were used as colorimetric spot tests for peroxidase.

Peroxidase activity was measured by adding a saturated aqueous solution of benzidine (0·04%; 5 ml.) to a gum solution diluted to 25 ml. Hydrogen peroxide (20 vol.; 0·05 ml.) was then added and after 5 min. the resulting blue was measured with an EEL colorimeter (Ilford no. 608 filter, peak transmission 680 mμ) with diluted gum solution as a blank.

Determination of reducing substances in plum-fruit tissues. Fruits were selected in July and cylindrical pieces of tissue removed from gumming and non-gumming mesocarps with a cork borer (no. 4). The tissue was disintegrated with a spatula and individually extracted three times with small volumes (10 ml.) of boiling methanol. Each extract was poured through a weighed sintered-glass funnel (medium porosity) and, finally, the tissue residues were transferred quantitatively to the funnels, washed with hot methanol (20 ml.) and dried to constant weights at 100°. The methanolic extracts were concentrated to syrups, then diluted with water to 10 ml. and small portions were analysed for reducing sugars by the Somogyi (1945) and benzidine methods (Jones & Pridham, 1954). Examination of these extracts on paper chromatograms showed that the main reducing component was glucose. The extracts were then separated on paper chromatograms and the glucose was determined with benzidine.

Respiration of plum-fruit tissues. Cylinders (1 cm. × 1 cm.) of mesocarp tissue were cut from healthy plum fruit and from gumming fruit (tissues adjacent to the 'lesion') with a cork borer. Respiration measurements were by the manometric method of Dixon (1951).

Examination of plum-fruit tissues for micro-organisms. Gumming fruits were surface-sterilized with ethanol, the nodules carefully removed and pieces of tissue from beneath were plated on to a nutrient-agar medium [Lab Lemco (10 g.) (Oxo Ltd.), peptone (10 g.), NaCl (5 g.), agar (20 g.) and water (1 l.)]. The plates were examined after incubation at 25° for 48 hr.

Enzyme preparations. 'Carterzymes' ADFP 3 and C.Z. 103 were supplied by H. W. Carter and Co. Ltd., Coleford, Gloucestershire, and enzyme 19 A.P. was obtained from Rohm and Haas, Co., Philadelphia, Pa., U.S.A.

RESULTS

Anatomical observations

Observation of plum trees in various orchards showed that nodules of gum were frequently present on the bark of the trunks and branches of the trees, particularly if the tissues had been damaged mechanically or if signs of disease were present as, for example, 'die-back'. Gum nodules were less common on the surface of the fruit, but these appeared much later in the season than those on the bark. Fruit with gum nodules at the distal end more often contained internal pockets of gum than those fruit with gum nodules along the ventral suture or other parts of the epicarp.

Microscopic examination of the cut surfaces of plums (picked in July) free from signs of external gummosis showed small globules of gum exuding from the severed ends of the vascular bundles rather than from the parenchymatous tissue. Initially these globules were colourless and mobile, but on standing they rapidly darkened and became highly viscous. In an atmosphere of nitrogen the globules remained colourless, and the increase in viscosity of of the gum was inhibited by a water-saturated atmosphere.

Sections through the mesocarp which were stained with a mixture of Congo red and chrysoidin revealed the presence of gum in the cells adjacent to the vascular bundles and also in the phloem itself. Gum nodules were invariably produced when the fruit epicarp was punctured with a sterile needle. The time taken for the gum to appear after the skin was punctured varied from approximately 1 hr. to 2–3 days. The exudation occurred with fruit on the tree and with 'picked fruit'.

Gum in the branches and trunks of trees appeared to be associated with the bast and rarely penetrated into the wood.

An examination of gumming 'lesions' on plums in an orchard showed that various bacteria and fungi were associated with the tissues directly

beneath the external gum nodules, but the mesocarp tissues adjacent to internal pockets of gum were invariably sterile. It is probable that in the majority of cases the micro-organisms found beneath the nodules were saprophytic rather than parasitic.

Sugars of the fruit

Glucose appeared to be the main reducing substance present in the mesocarp tissues of both gumming and non-gumming fruit. Quantitative analysis (Table 1) showed that in the latter the glucose concentration was approximately 40% higher than in the former. Traces of D-xylose, which were tentatively identified by Hay & Pridham (1953) in the mesocarp, have now been confirmed by a comparison of the X-ray powder photograph of an authentic dibenzylidenedimethylacetal derivative of D-xylose (Breddy & Jones, 1945) with a similar derivative of the xylose isolated from the fruit.

In addition to glucose and xylose, sucrose, fructose and traces of raffinose were detected on paper chromatograms (cf. Bradfield & Flood, 1950).

Measurement of respiration showed that normal tissues had a significantly higher rate than gumming tissues (Table 2) and that the R.Q. values for the latter were higher than those for the normal tissue.

Table 1. *Reducing power and concentration of glucose in gumming and non-gumming fruit-mesocarp tissues*

Tissue		Reducing power (as % of glucose, based on dry wt. of tissue): Benzidine method	Somogyi method	Glucose (% based on dry wt. of tissue)
Non-gumming	1	68·8	57·7	—
	2	60·8	59·3	48·2
Gumming	1	34·2	30·7	27·0
	2	35·6	31·4	28·4

Table 2. *Respiration of gumming and non-gumming fruit-mesocarp tissues*

Tissue		Gas (μl./g. dry wt.): CO_2	O_2	R.Q.
Non-gumming	1	386	188	2·1
	2	355	187	1·9
	3	353	191	1·9
Average value		365	189	1·9
Gumming	1	306	79	3·9
	2	381	145	2·6
	3	280	103	2·7
Average value		322	109	3·0

R.Q. values for both types of tissue were greater than unity.

Some preliminary experiments designed to test the influence of gums on the growth of micro-organisms showed that the crude materials were bacteriostatic. A pure culture of an unidentified bacillus was isolated from the cotyledons of gumming almonds. When this organism was grown on a nutrient-agar plate which contained small nodules of plum-bark gum (surface-sterilized with ethanol) embedded into the medium, zones of growth inhibition were evident around each nodule. Bacilli growing on the boundaries of these zones were pleomorphic and many spore forms were present.

Chemical constitution of Victoria-plum gums

Paper-chromatographic examination of the hydrolysates of bark, fruit and stone gums showed that glucuronic acid, galactose, mannose, arabinose, xylose and rhamnose were present in all the gums, and in similar proportions with the exception of mannose, which appeared to be more prevelant in the bark gum.

Hydrolysis of fruit-surface gum followed by separation of the component sugars afforded crystalline specimens of D-galactose ($[\alpha]_D + 79°$; m.p. and mixed m.p., 156°); L-arabinose ($[\alpha]_D + 102°$; m.p., 149°, mixed m.p. 148°) and L-rhamnose hydrate ($[\alpha]_D + 10·5°$; m.p. and mixed m.p. 86°). D-Mannose ($[\alpha]_D + 11·9°$) and D-xylose ($[\alpha]_D + 20·7°$) were obtained as syrups and were characterized as the phenylhydrazone (m.p. and mixed m.p. 181–182°) and dibenzylidenedimethylacetal derivative (m.p. and mixed m.p. 190°) respectively.

The uronate fraction obtained from the hydrolysate was shown to contain at least three acidic oligosaccharides and two other compounds which co-chromatographed with glucurono-γ-lactone and 4-*O*-methylglucuronic acid respectively. The former was obtained as a crystalline compound (m.p. and mixed m.p. 173°). The mixed oligosaccharides on acid hydrolysis yielded galactose, mannose, glucuronic acid and a trace of arabinose. Examination of the hydrolysis products of the reduced oligosaccharides showed the presence of glucose, galactose, mannose, arabinose and 4-*O*-methylglucose.

Mild acid hydrolysis of hexuronic acid-containing polysaccharides with 100% recovery of the liberated monosaccharide components is rarely achieved, owing to the stability of the glycuronosidic bonds, and with strong mineral acids marked destruction of the monosaccharides liberated occurs. The conditions necessary for the optimum hydrolysis of Victoria-plum gum to a mixture of monosaccharides and aldobiouronic acids with N-H_2SO_4 were 4–6 hr. at 100° in a sealed tube. This was revealed by following the change in optical rotation during

Table 3. *Relative proportions of monosaccharides liberated on hydrolysis of plum gums in an autoclave*

Hydrolysis (15 lb./in.², 120°) was for 1 and 2 hr. respectively, with N-H_2SO_4. The values for 1 hr. hydrolysis are averages of two determinations; those for 2 hr. hydrolysis are averages of four determinations.

	D-Galactose		D-Mannose		L-Arabinose		D-Xylose	
Gum	1 hr.	2 hr.	1 hr.	2 hr.	1 hr.	2 hr.	1 hr.	2 hr.
Bark	1·0	1·0	0·18	0·71	0·66	1·51	0·27	0·24
Fruit surface	1·0	1·0	0·18	0·11	0·98	0·88	0·22	0·21

hydrolysis. In order to speed the routine analysis of the gum polysaccharides, attempts were made to shorten the hydrolysis time by carrying out the hydrolysis in an autoclave at 120° with N-H_2SO_4.

The change in optical rotation of solutions of various monosaccharides after varying periods of time was studied. They were extensively degraded under these conditions; after 2 hr., 33 % of L-arabinose and 22 % of D-galactose were lost (assuming that the degradation products had little or no optical activity). The technique is therefore of little use in determining absolute concentrations of monosaccharides liberated from polysaccharides, but it is useful when rapid qualitative comparative results are needed, particularly for hexuronic acid containing materials.

Aqueous solutions of plum gums readily undergo autohydrolysis because of the lability of the L-arabinose residues, which are presumably present as non-reducing end groups in the furanose form. For this reason, a comparative quantitative analysis was carried out with the crude gums to alleviate the loss of monosaccharides by hydrolysis during purification.

Analysis for uronic acid anhydride revealed that the fruit-surface gum contained less hexuronic acid (13 %) than the bark gum (18 %) when corrections were made for the ash and water content in both.

The relative proportions of D-mannose, L-arabinose and D-xylose to D-galactose were determined after acid hydrolysis of bark and fruit-surface gums under identical conditions (Table 3). After hydrolysis for 1 hr. there was little difference in the composition of the hydrolysis mixtures of the two types of gum except that a slightly higher concentration of L-arabinose had been liberated from the fruit-surface gum. However, after hydrolysis for 2 hr. it was evident that the bark gum contained a much higher concentration of L-arabinose and D-mannose than the fruit-surface gum.

When the hydrolyses (100°, sealed tube) were followed by measuring the optical rotations of the reaction mixtures, the results were similar for both types of gum. As the hydrolyses proceeded the reaction mixtures were examined on paper chromatograms at varying intervals. With both gums L-arabinose was liberated a few minutes after the reaction had started, and after 30 min. L-arabinose, L-rhamnose, D-galactose, D-xylose and three acidic oligosaccharides were detectable, together with traces of D-mannose. The last-named sugar gradually increased to a maximum after about 8 hr. After 5 hr. glucuronic acid was also detectable, its appearance on chromatograms coinciding with the gradual fall in intensity of the oligosaccharide spots. There appeared to be less hexuronic acid liberated from the fruit-surface gum than from the bark gum and the relative concentrations of the acidic oligosaccharides varied for the two gums.

Table 4. *Periodate oxidation of plum gums*

	Gum	Wt. of gum consuming 1 mole of periodate (g.)	Wt. of gum liberating 1 mole of formic acid (g.)
Purified	Bark	158	717
	Fruit surface	219	1225
Crude	Bark	179	726
	Fruit surface	233	1003

Attempts to degrade the polysaccharide of plum-bark gum to lower molecular-weight fragments with aqueous solutions of commercial enzyme preparations (i.e. enzyme 19A.P., Carterzyme ADFP3 and Carterzyme C.Z.103) failed.

Periodate oxidations of crude and purified specimens of both types of gum were carried out, and the uptake of periodate and liberation of formic acid were determined at various intervals of time. A comparison of the results for both purified and crude gums (Table 4) shows that purification by alcoholic precipitation had little degradative effect on the polysaccharides, differences in results probably being due to the varying ash content of the crude gums.

Electrophoresis

The potentialities of the Tiselius electrophoresis apparatus for the separation of mixtures of acidic polysaccharides were examined. Preliminary experiments showed that plum (var. Czar)-bark

gum could be separated from gum acacia with 0·2 M-borate–KCl buffer, pH 7·8. Similarly, a mixture of sugar-beet araban and plum-bark gum could be resolved with 0·07 M-phosphate buffer, pH 7·0.

Electrophoresis of plum-bark gum in several buffers (0·2 M-borate–KCl, pH 7·8; 0·07 M-phosphate, pH 7·0; 0·1 M-acetate, pH 6·0; 0·1 M-citrate, pH 3·0) suggested that it was homogeneous, as single peaks were observed in all cases. The boundaries migrated towards the positive electrode and in the borate buffer the rate of movement was calculated as $3{\cdot}08 \times 10^{-5}$ cm²/v/sec. However, attempts to separate artificial mixtures of fruit-surface gum and bark gum in borate buffer failed.

Chromatography

Paper-chromatographic examination of cold methanolic extracts of various plant gums showed that in many cases traces of mono- and oligosaccharides were present (Table 5). At least three oligosaccharides were present in cherry-bark gum and small amounts (5–30 mg.) of each were isolated on Whatman no. 3MM paper with solvent (1). Each was completely hydrolysed to arabinose when heated with 0·01 N-H_2SO_4 for 1·5 hr. at 95°. The $R_{galactose}$ values of the three sugars in solvent (1) were 1·2, 0·65 and 0·30, and an application of the rule of Consden, Gordon & Martin (1944) suggested that oligosaccharides were a homologous series consisting of arabinobiose, arabinotriose and arabinotetraose respectively. The suspected disaccharide ($R_{galactose}$ 0·30; $[\alpha]_D^{25} + 112°$) gave a phenylosazone in small yield (m.p. 190°).

The three oligosaccharides detected in gum arabic had $R_{galactose}$ values (in solvent 1) of 0·46, 0·53 and 0·73. A small amount of the fastest-moving material, again isolated by partition on thick paper, was shown to be stable to 0·01 N-H_2SO_4 (95°; 1 hr.) but was completely hydrolysed to arabinose with N-H_2SO_4 under the same conditions. This oligosaccharide also co-chromatographed with an authentic specimen of 3-*O*-β-L-arabinopyranosyl-L-arabinose. Cold ammoniacal methanol also extracted galactose, arabinose, rhamnose and the above-named oligosaccharides from gum arabic.

Examination of a number of specimens of plum gum and fruit tissues for sugar phosphates yielded negative results. Inorganic phosphate only was detected.

The residues obtained from gum after strong acid hydrolysis yielded on oxidation with alkaline nitrobenzene a compound which co-chromatographed with vanillin and was detected on paper chromatograms with a 2:4-dinitrophenylhydrazine spray reagent. Both bark gum and fruit-surface gum reacted similarly.

Table 5. *Free sugars detected in plant gums*

Gum	Sugars detected
Cherry bark	Galactose, arabinose and three oligosaccharides
Arabic	Galactose, arabinose, rhamnose and three oligosaccharides
Almond bark	Arabinose and xylose
Plum bark (var. Cose Late Red)	
Sample I	Glucose, fructose and one oligosaccharide
Sample II	Sucrose, glucose and arabinose
Plum bark (var. Victoria)	Glucose, arabinose, xylose and two or three oligosaccharides

Phenolic and amino compounds

Paper chromatographic examination of the ether and ethyl acetate extracts of an aqueous solution of plum-bark gum showed the presence of four main components (*D*, *E*, *F* and *G*) with the properties listed in Table 6. Traces of these compounds were also detected in the residual aqueous solution. The properties of compound (*F*) were identical with those of phloroglucinol, both compounds giving similar u.v. spectra and having the same chromatographic behaviour. Phenolic glycosides appeared

Table 6. *Properties of phenolic compounds present in plum gums*

	Ether extract		Ethyl acetate extract	
Compounds	*D*	*E*	*F*	*G*
R_F [solvent (6)]	0·79	0·90	0·68	0·63
R_F [solvent (4)]	0·79	0·87	0·72	0·66
Fluorescence with u.v. light + NH_3	Pale blue	Pale blue	Blue	Pale blue
Colour with diazotized *p*-nitroaniline–Na_2CO_3 spray reagent	Brown	Brown	Red–brown	Yellow
Reaction with diazotized sulphanilic acid spray reagent	Orange	—	Orange	—
U.v.-absorption maxima (mμ)	257	—	268	—

to be absent from the gum. Fruit-surface gum was shown to have a chromatographically similar phenolic content. Plum-bark gum gave a positive Mitchell's test for tannins (Mitchell, 1923).

Analysis of several samples of plum gum showed that they all contained a small amount of nitrogen (0·3–0·6 %). No free amino acids could be detected, but after mild acid hydrolysis of the gum several compounds, which gave a blue with ninhydrin, were liberated.

Enzymes

Qualitative spot tests revealed the presence of peroxidase activity in both bark gum and fruit-surface gum. Preliminary experiments indicated that the benzidine–H_2O_2 spot test could be adapted as a quantitative method and that for the gum peroxidase there was a linear relationship between the blue produced and the weight of gum used. With this method it was shown that a higher peroxidase activity was associated with bark gum than with fruit-surface gum (Fig. 1). The peroxidase in solutions of gum (2 ml.; 1 %, w/v) was completely inactivated by heating for 1 min. in a boiling-water bath.

Phenolase activity in gums, as shown by the reaction with benzidine in the absence of H_2O_2 or by the spot test with catechol, was very small. The tissues of the fruit and bast, however, contained a relatively high concentration of phenolase.

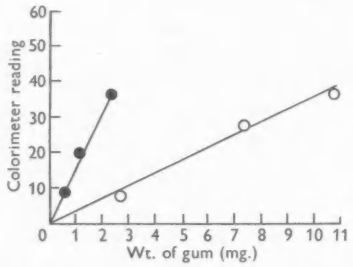

Fig. 1. Peroxidase activity in plum-bark gum (●) and fruit-surface gum (○).

Table 7. *Inorganic cations present in plum-stone gum and fruit-surface gum*

Cation	Fruit-surface gum	Stone gum
Ca	+ + +	+ + +
Mg	+ + +	+ + +
Na	+ +	+ +
K	+ +	+
Al	+ +	+ +
Sr, Ba, Be, Fe	–	–

An examination of the inorganic cations present in fruit-surface gum and stone gum of plums suggested that the two types of gum were essentially similar in mineral content. The results of this analysis are given in Table 7.

DISCUSSION

Our observations support the theory that gums are normal plant products although micro-organisms may stimulate the formation of excess of gum. Frequently excessive gum formation cannot be explained by mechanical injury or the presence of micro-organisms.

Microscopic examination of immature plum fruits showed that small amounts of gum were present in apparently sterile tissues. The hardening of the globules of gum was probably due to dehydration and not polymerization, because in a water-saturated atmosphere this increase in viscosity was prevented. In the absence of oxygen the globules remained colourless, thus suggesting that the dark colour of many gums is due to the oxidation of phenolic compounds.

The soluble sugars present in the mesocarp tissues of plum fruit included sucrose, glucose, fructose and small amounts of raffinose and xylose. All of these compounds were detected in both gumming and non-gumming fruits, but in the latter the concentration of glucose was significantly higher than in tissues showing excessive gummosis. Hexoses will be consumed in normal metabolic processes, and with gumming tissues it is probable that, in addition hexoses will also be utilized for the biosynthesis of gum polysaccharide. This may lead to their depletion, but it seems improbable that this alone would account for the fall in respiration. It seems more likely that the differences in respiration and the higher R.Q. obtained with the gumming tissues are due to the formation of a gum barrier which interferes with gaseous exchange, although partial anaerobiosis due to the thickness of the tissues would account for the high R.Q. values obtained in all cases.

It has often been stated (e.g. Hirst, 1949) that the function of the gum in the plant is to seal mechanical wounds and to 'wall-off' pockets of infection. Experiments with plum gum showed that commercial enzyme preparations, which will hydrolyse pectin and hemicelluloses, failed to degrade the gum polysaccharide. Such preparations consist of a highly reactive mixture of fungal carbohydrases. Some evidence was obtained for the presence of active bacteriostatic materials in the gum. The zones of growth inhibition which were observed when gum nodules were introduced into plate cultures of the bacillus may have been caused by the simple phenolic constituents of the gum or by

tannins. Another possibility is that the gum peroxidase has an antimicrobic action, since proteins can be oxidized and certain enzymes inactivated by this enzyme in the presence of hydrogen peroxidase (Sizer, 1953).

The monosaccharide composition of bark gums from several species of Rosaceae have been examined, e.g. cherry (Jones, 1939), damson (Hirst & Jones, 1938), egg plum (Hirst & Jones, 1947), peach (Jones, 1950) and almond (Brown, Hirst & Jones, 1948), but little has been reported on the composition of fruit-surface gums from any plant family. The Rosaceae bark gums have all been observed to contain D-glucuronic acid, D-galactose, L-arabinose and D-xylose, and some also contain D-mannose and L-rhamnose. The homogeneity of the gum polysaccharides is questionable, particularly in view of the recent work of Heidelberger, Adams & Dische (1956), who used immunological techniques to resolve gum arabic into two fractions with different monosaccharide compositions. With bark gum from egg plum, the gum nodules from different trees and varying locations have been observed to contain a constant monosaccharide composition (Hirst & Jones, 1947). Stone (1890) compared the galactose and pentose contents of the fruit-surface gum and bark gum of peach and concluded that they were similar, although the analytical methods used are open to criticism.

Analysis of plum-bark gum and fruit-surface gum revealed that the monosaccharides in both were essentially the same (4-*O*-methylglucuronic acid was not detected in bark gum, however), but the bark gum contained a greater proportion of hexuronic acid, mannose and arabinose than did the fruit-surface gum (Table 3). The differences in analytical data obtained for the 1 and 2 hr. hydrolyses can be explained by assuming that larger proportions of L-arabinose and D-mannose in the bark gum are linked to the glycosidic carbons of hexuronic acids than in the fruit-surface gum. Acid-stable aldobiouronic acids would be among the first products of hydrolysis and these units would be cleaved only by vigorous hydrolytic conditions. Support for this theory is given by the fact that acidic oligosaccharides were detected in the partial hydrolysis products of both types of gum.

The relative proportions of the monosaccharides present after hydrolysis for 1 hr. suggest that some of the D-mannose and L-arabinose units exist in a similar state of combination in both gums.

Periodate oxidation of the two gums also showed a distinct structural difference (Table 4). The results indicate that the bark-gum polysaccharide has a greater proportion of pyranosyl non-reducing end groups than the fruit-surface gum.

Qualitative analysis of the stone gum showed that it had a monosaccharide composition similar to that of the fruit-surface gum. Quantitative analysis was not possible because of the difficulty in isolating the gum free from pectin and other fruit polysaccharides. The stone gum was less soluble in water than the fruit-surface gum and it was suspected that this was due to a difference in the inorganic content of the two gums. Paper-chromatographic analysis, however, showed that the same cations were present in essentially similar proportions (Table 7). The solubility difference may be a purely physical phenomenon rather than structural differences in the macromolecules.

Colvin, Cook & Adams (1952) and Isherwood (1949) have studied the electrophoresis of polysaccharides. The former workers were able to separate an artificial mixture of sodium alginate and pectin and Isherwood was able to resolve mixtures of hemicelluloses obtained from pear cell wall. Our own preliminary experiments indicated that plant gums could be examined electrophoretically, but the length of time used for separations was limited owing to the rapid diffusion of the boundaries. With a phosphate buffer, pH 7·0, plum-bark gum was readily separated from sugar-beet araban and it could also be separated from gum arabic with borate buffer, pH 7·8. Bark gum appeared to be homogeneous in a variety of buffers of varying pH, but despite the obvious differences in the structures of bark gum and fruit-surface gum, an artificial mixture of the two could not be resolved electrophoretically. The homogeneity of plum gums is therefore still doubtful.

Small amounts of mono- and oligo-saccharides were detected in a number of different gums. Free sucrose, glucose, fructose and xylose were detected in the plant tissues; hence the gums could easily have become contaminated with these sugars. Arabinose may have arisen by autohydrolysis of arabofuranose end groups, which are common in many plant polysaccharides. In view of the mild extractive conditions, however, arabinose and the other oligosaccharides probably arose as a result of photochemical degradation of the gum polysaccharides whilst on the tree and are not substrates for gum biosynthesis.

The lability to acid of the arabinose oligosaccharides extracted from cherry gum suggested that arabofuranose units were present (Hirst, 1949). The disaccharide was probably 3-*O*- or 4-*O*-L-arabofuranosyl-L-arabinose. A $1 \rightarrow 5$ linked disaccharide is improbable owing to the high positive optical rotation. 2:5-Di-*O*-methyl-L-arabinose has been isolated from methylated cherry gum (Jones, 1947), and this could arise from $1 \rightarrow 3$ linked arabofuranose units.

The available evidence suggests that one of the oligosaccharides extracted from gum arabic is 3-*O*-β-L-arabopyranosyl-L-arabinose, a disaccharide which has been isolated from the partial hydrolysis products of several gums (Andrews, Ball & Jones, 1953; Charlson, Nunn & Stephen, 1955). The detection of this disaccharide in gum arabic is of interest in relation to the structural studies of Dillon, O'Ceallachain & O'Colla (1953), who state that one of the side chains in the gum-polysaccharide molecule must contain two arabinose units, in addition to the galactose end group, instead of a single unit as was originally suggested (Jones & Smith, 1949).

The small amount of nitrogen present in plum gums is probably all proteinaceous. Phenolase (oxidase) and peroxidase have been observed in gum arabic (Laursen, 1932) and various colorimetric tests have confirmed the presence of peroxidase in plum gums, although the phenolase activity appeared to be low.

The bark gum is invariably darker in colour than the fruit-surface gum and this may be due to the higher peroxidase activity in the former, which results in an increase in coloured phenolic oxidation products. In this connexion it is interesting that oxidized phenolic compounds can be antimicrobic (R. J. W. Byrde, A. H. Fielding & A. M. Williams, unpublished results).

Paper chromatography showed that many of the phenolic materials found in the fruit-mesocarp tissues of plum were not present in bark gum or fruit-surface gum. The fruit tissues contained a large number of compounds which, on paper chromatograms, reacted with the diazotized *p*-nitroaniline–sodium carbonate spray reagent and fluoresced under u.v. light. Evidence was also obtained for the presence of a number of phenolic glycosides. The gums, however, contained only a small number of phenolic constituents and phenolic glycosides were not apparent. It is tempting to suggest that the phenolic glycosides are utilized for the biosynthesis of gum polysaccharide, with the liberation of the phenolic aglycones. There is no direct evidence for this hypothesis, however.

With regard to the biosynthetic route of gum-polysaccharide formation the analytical results show that the plum-bark gum and fruit-surface gum have different compositions and may therefore be formed by different processes, although similar repeating units, synthesized by the same pathways, could be present in both gums.

SUMMARY

1. The composition, function and formation of gum has been studied with particular reference to the plum tree (var. Victoria).

2. The polysaccharide moiety of the gum exuded on the fruit surface was composed of D-galactose, D-mannose, L-arabinose, D-xylose, L-rhamnose, glucuronic acid and probably traces of 4-*O*-methylglucuronic acid. The bark gum was similarly constituted but contained higher proportions of D-mannose, L-arabinose and hexuronic acid. Periodate oxidation of the two types of gum suggested structural differences though their electrophoretic mobilities were identical.

3. Crude bark gum and fruit-surface gum contain the enzyme peroxidase, phenolic compounds and a 'lignin-like' substance and some inorganic material. Gums from several different species of plants were observed to contain small amounts of mono- and oligo-saccharides.

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